Synthesis of jasmonates and derivatives to study plant signaling: Activation, translocation and shutdown mechanisms

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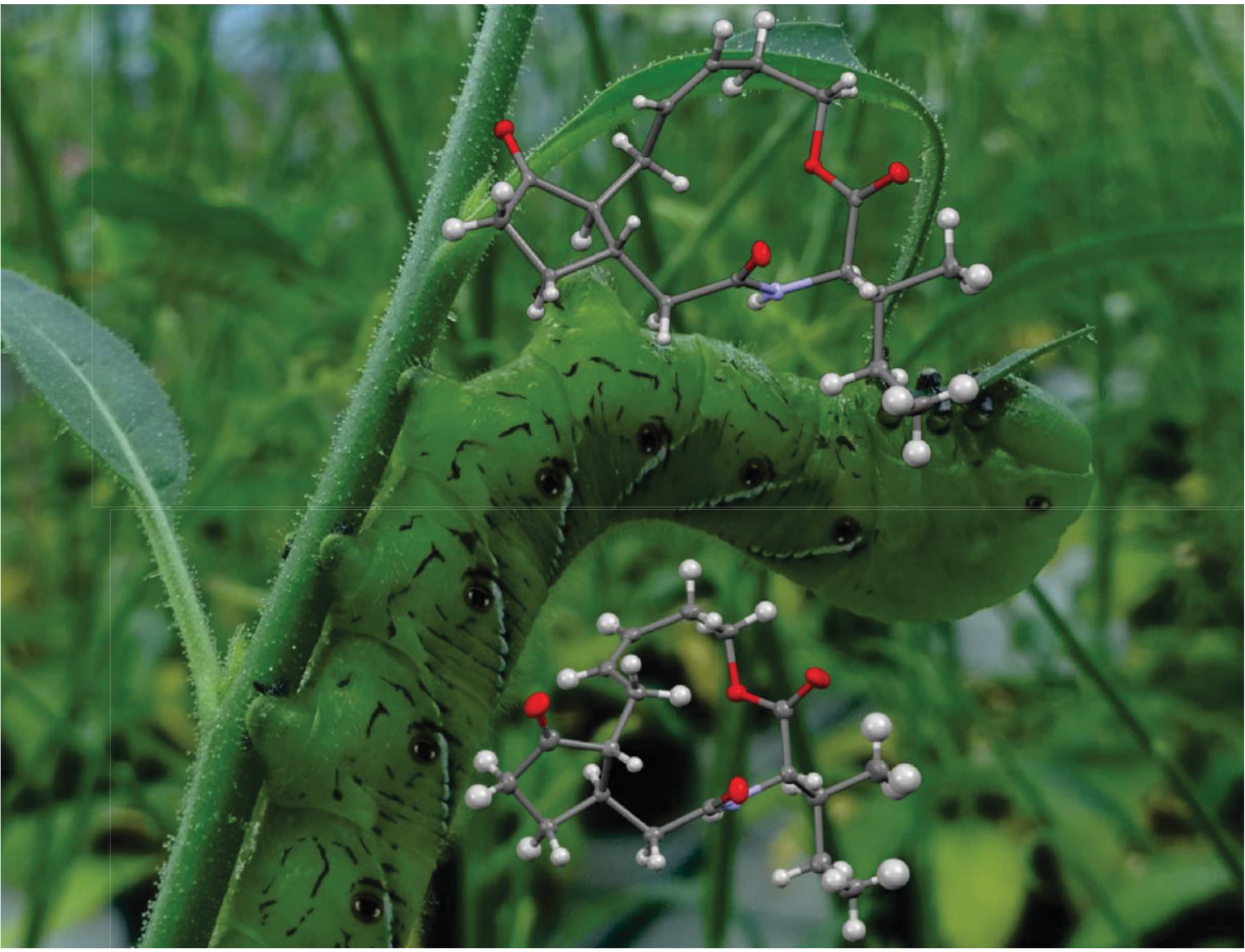
“I do not know what I may appear to the world, but to myself I seem to have been only like a boy playing on the sea-shore, and diverting myself in now and then finding a smoother pebble or a prettier shell than ordinary, whilst the great ocean of truth lay all undiscovered before me.”

Sir Isaac Newton

"…to my family!"

Synthesis of jasmonates and derivatives to study plant signaling: Activation, translocation and shutdown mechanisms



Content

Abbreviations

12,13-EOT	12,13–epoxyoctadecatrienoic acid
13-HPOT	(13*S*)-hydroperoxyoctadecadienoic acid
AcOH	Acetic acid
AcOEt	Ethyl acetate
ACX	Acyl-CoA oxidase
AOC	Allene oxide cyclase
AOS	Allene oxide synthase
ATP	Adenosine 5'-triphosphate
AUX-IAA	Auxin/indole-3-acetic acid
bp	Base pair
ca.	Circa, approximately
CAMTA	Ca^{+2}-calmodulin-binding transcription activator proteins
cDNA	Complementary DNA
CoA	Coenzyme A
CM	Cross metathesis
CML	Calmodulin-like proteins
COI1	Coronatine insensitive 1
CKs	Cytokinins
CT	Computed tomography
CYP	Cytochrome P450
d	Day(s)
DAD1	Defective in anther dehiscence 1
DELLA	Negative regulators of gibberellin (GA)
DNA	Deoxyribonucleic acid
EI	Electron impact
ER	Endoplasmic reticulum
ESI	Electro-Spray-Ionization
ET	Ethylene
EtOH	Ethanol
GA	Gibberellic acid
h	Hour(s)
HCl	Hydrochloric acid
HTA	Hexadecatrienoic acid
JA	Jasmonic acid

JA-Ile	*N*-jasmonoyl-L-isoleucine
JAR1	Jasmonic acid-amido synthetase
Jas	Jasmonate associated domain
JAZ	Jasmonate ZIM domain
JMT	JA methyltransferase
JRG	Jasmonate responsive gene
KAT	L-3-ketoacyl CoA thiolase
LA	α-linolenic acid
LC-MS	Liquid chromatography mass spectrometry
LoF	Loss-of-function
LOX	Lipoxygenase
MAMP	Microbe-associated molecular patterns
MeJA	Methyl jasmonate
MeOH	Methanol
MFP	Multifunctional protein
MRI	Magnetic resonance imaging
MS	Mass spectrometry
m/z	Mass-charge ratio
NMR	Nuclear magnetic resonance
NPR1	Non-expressor of pathogenesis related 1
OPC-8:0	8-((1S,2S)-3-oxo-2-((Z)-pent-2-en-1-yl)cyclopentyl)octanoic acid
OPCL1	OPC-8:0-CoA ligase 1
OPDA	*cis*-(+)-12-oxo-phytodienoic acid
OPR3	OPDA-reductase 3
OS	Oral secretion
PAMP	Pathogen-associated molecular pattern
PCR	Polymerase chain reaction
PET	Positron emission tomography
PETIS	Positron-emitting tracer imaging system
qPCR	Quantitative real time PCR
RNA	Ribonucleic acid
ROS	Reactive oxygen species
SA	Salicylic acid
SAR	Structure-activity relationship
SRL	Sensor relay proteins
SRP	Sensor responder proteins

TF	Transcription factor
THF	Tetrahydrofuran
TPC1	Two pore channel 1 protein
UHPLC	Ultra-high performance liquid chromatography
V_m	Voltage of the membrane
Vol.	Volume
v/v	Volume / volume
WT	Wild type

Summary / Zusammenfassung

Plants are organisms that are fixed in space during their entire life. The rapid adaptation to environmental cues is critical for plant survival and continuity of the species. Light, nutrients and chemical signals are among the most important cues plants can perceive. Chemical signals can initiate complex interaction networks which are governed to a high extend by phytohormones. Plant hormones can include a biochemical and physiological reprograming affecting processes like photosynthesis, flowering, shoot or root development and the production of defense compounds.

Phytohormones are among the most important chemicals present in plant tissues. Within this class of compounds, jasmonates (JAs) are fatty acid derivatives that play a key role in regulating both biotic and abiotic stress responses, as well as processes related to plant growth and development all over the plant kingdom. Jasmonic acid (JA), methyl jasmonate (MeJA), and *N*-jasmonoyl-L-isoleucine (JA-Ile) are among the best characterized members of the JAs family. Several studies have shown that JAs can be modified (e.g., glycosylated, sulfonated, and hydroxylated) *in planta* to form derivatives of great importance for the fine tuning of plant physiological responses.

The main goal of my thesis was to study different aspects of JAs signaling in plants with the help of endogenous JAs and JA-derivatives synthesized chemically. In particular, I studied the relationship between the Calcium II (Ca^{2+}) and the JA-signaling pathways as an early event in plant signaling. I also reported on late events in JA signaling like the metabolism and systemic translocation of a fluorinated mimic of the JA precursor OPC-8:0 in *Arabidopsis thaliana* plants. Finally, I focused on the perception and inactivation mechanisms of the biologically active jasmonate JA-Ile. The main outcomes of my work are presented below.

Manipulation of Ca^{2+} signaling affects JA-Ile accumulation and signaling

In line with previous studies, I corroborated the close relationship between cytosolic free Ca^{2+} ($[Ca^{2+}]_{cyt}$) elevation and JA signaling. I reported that the antibiotic neomycin selectively blocks the $[Ca^{2+}]_{cyt}$ elevation induced by oral secretions of *Spodoptera littoralis* larvae in *A. thaliana* plants. This impaired

response inhibits the accumulation of the bioactive JA-Ile in the leaves, but not the increase of JA levels. Accordingly, neomycin treatment reduced the expression level of the JA responsive genes (JRG) *VSP2* and *LOX2*. Low levels of JA-Ile were correlated with an increase in the expression of the CYP94B3 enzyme, which ω-hydroxylates JA-Ile causing its inactivation. These findings suggested that the $[Ca^{2+}]_{cyt}$ elevation specifically controls JA-Ile accumulation and signaling, offering new insights into the role of Ca^{2+} signaling in plant defenses against insects. Furthermore, neomycin is presented as a valuable tool to investigate the link between Ca^{2+}- and JA-signaling, especially in plants species where the lack of mutants is still a drawback.

Fluorinated JAs as probes to study metabolism and signaling of endogenous compounds

There is a lack of tools to evaluate the spatial and temporal distribution of JAs in real time. Therefore, I explored the possibility of using a fluorinated jasmonate as a molecular probe to study metabolic processes of this molecule. I carried out the synthesis of 7F-OPC-8:0 – a fluorinated analog of the JA precursor OPC-8:0. The fluorinated compound behaves similarly to the endogenous oxylipin in *A. thaliana* plants. 7F-OPC-8:0 induces both the accumulation of jasmonates and the expression of JRG after its application to the leaves. By using UHPLC-MS/MS, I could detect metabolites derived from the β-oxidations of 7F-OPC-8:0 in leaves extracts. This was direct evidence that 7F-OPC-8:0 is metabolized *in vivo* similarly to the endogenous OPC-8:0. Furthermore, I successfully employed 7F-OPC-8:0 as a probe to show its translocation from a damaged leaf to undamaged systemic leaves. The last suggests that the JA precursor OPC-8:0 may act as a mobile signal which induces systemic defense responses. My study outlined the potential of fluorine-chemistry to study jasmonates – and perhaps other phytohormones and plant lipid derivatives – metabolism and signaling.

Rational design of synthetic JA-derivatives to study the perception and inactivation mechanisms of the biologically active jasmonate JA-Ile

In this study I explored the possibility of manipulating the JA-Ile signalling pathway by introducing tailored modifications on the ligand. Inspired by the chemical

structure of jasmine ketolactone ─ a naturally occurring jasmonate ─, and based on the structure of the JA-Ile receptor, I synthesized two diastereomeric macrolactones derived from JA-Ile. These compounds enabled me to further explore potential biological activities of rationally-designed JA-Ile derivatives. I tested the ability of the lactones to elicit nicotine production in *Nicotiana attenuata* plants. Both macrolactones induced nicotine accumulation to a similar extent as MeJA does. Interestingly, the JA-Ile-lactone which has a non-natural configuration induced the highest nicotine contents. These observations prompted me to investigate the JA-Ile inactivation mechanism upon ω-hydroxylation using *A. thaliana* as a model. With the help of 12-modified JA-Ile derivatives (including the above mentioned lactones), I found that the JA-Ile-lactones activate JRG expression similarly to JA-Ile in *A. thaliana*. This result indicates that the free carboxyl group of JA-Ile is not essential for this molecule to induce JRG expression. Furthermore, a set JRG were differentially expressed depending on the configuration of the JA-Ile-lactone. Moreover, my results suggested that impaired activity of JA-Ile after ω-hydroxylation is due to steric hindrance caused by the introduced hydroxyl group in the binding pocket of the JA-Ile receptor. Molecular modelling which will provide further insights into the JA-Ile perception and inactivation mechanisms are in progress.

In summary, the data presented in my thesis constitute direct evidence of the possibility of study and manipulate the JA signalling pathway by using small molecules. I have introduced new knowledge of the molecular mechanism of JA signalling ─ from early events like Ca^{2+}-induced JA-Ile burst, until late events like the switch-off of the jasmonate signal. This work contributed to the better understanding of a major plant hormone pathway.

Summary / **Zusammenfassung**

Pflanzen sind Organismen, die ihr Leben lang an einen Standort gebunden sind. Eine schnelle Anpassung an sich verändernde Umweltbedingungen ist daher sowohl für das Überleben der einzelnen Pflanze als auch den Fortbestand der Art entscheidend. Licht, Nährstoffe und chemische Signale zählen zu den wichtigsten Faktoren, die Pflanzen wahrnehmen können. Chemische Signale lösen komplexe, vernetzte Antworten aus, die hauptsächlich durch Phytohormone gesteuert

werden. Diese komplexen Reaktionen beinhalten biochemische und physiologische Veränderungen, die sich auf Prozesse wie die Photosynthese, die Blütenbildung, die Entwicklung von Spross und Wurzeln und die Produktion von Abwehrstoffen auswirken.

Phytohormone zählen zu den wichtigsten chemischen Verbindungen in pflanzlichen Geweben. Im gesamten Pflanzenreich spielen innerhalb dieser Hormone die Jasmonate, eine Gruppe von Fettsäurederivaten, eine zentrale Rolle in der Regulation von Reaktionen auf abiotischen und biotischen Stress, sowie beim Pflanzenwachstum und der Entwicklung. Innerhalb der Jasmonat-Familie sind Jasmonsäure (JA), Methyljasmonat (MeJA), und Jasmonsäure-Isoleucin (JA-Ile) am besten untersucht. Verschiedene Studien zeigten, das Jasmonate *in planta* chemisch modifiziert werden können (z.B. durch Glykosylierung, Sulfonierung und Hydroxylierung). Die dabei entstandenen Derivate sind von großer Bedeutung für die Feinabstimmung der physiologischen Reaktionen der Pflanze.

Das Hauptziel meiner Arbeit war es verschiedene Aspekte von Jasmonatsignalwegen mit Hilfe von chemisch synthetisierten Jasmonaten und deren Derivaten zu untersuchen. Dafür untersuchte ich die Verbindung zwischen Calciumsignalen und Jasmonatsignalwegen, welche eine wichtige Rolle in der frühen Signaltransduktion einer Pflanze spielt. Außerdem erforschte ich den Metabolismus und die systemische Translokation eines fluorinierten Derivats des JA-Vorläufers OPC-8:0 in *Arabidopsis thaliana* Pflanzen. Abschließend konzentrierte ich mich auf die späten Ereignisse im Jasmonatsignalweg und untersuchte die Perzeption und den Inaktivierungs-mechanismus des biologisch aktiven Jasmonats JA-Ile. Die Hauptergebnisse meiner Arbeit sind im Folgenden aufgeführt.

Die Manipulation von Ca^{2+}-Signalen beeinträchtigt die JA-Ile-Akkumulation und die nachfolgenden Signale

In Übereinstimmung mit bisherigen Studien, konnte ich die enge Verbindung zwischen cytosolischen Calciumsignalen und Jasmonaten bestätigen. Ich zeigte, dass die Erhöhung des cytosolischen Calciums, angeregt durch das Oralsekret von *S. littoralis* Larven, durch das Antibiotikum Neomycin in *A. thaliana* inhibiert werden kann. Diese Störung des Calciumsignals verhindert die Akkumulation des

bioaktiven JA-Ile in den Blättern der Pflanze, jedoch verändert sie nicht die JA-Level. Dementsprechend ist die Expression der JA-Ile regulierten Gene *VSP2* und *LOX2* in mit Neomycin behandelten Pflanzen reduziert. Die geringen Mengen an JA-Ile gingen mit einer erhöhten Expression des CYP94B3 Enzyms einher, welches JA-Ile in ω-Position hydroxyliert und somit inaktiviert. Diese Ergebnisse weisen darauf hin, das Calciumsignale die JA-Ile-Akkumulation spezifisch kontrollieren und somit auch die nachfolgenden Signale, was uns neue Erkenntnisse über die Rolle von Calciumsignalen in der pflanzlichen Abwehr gegen Insekten liefert. Des Weiteren konnte gezeigt werden, dass Neomycin ein wertvolles Hilfsmittel zur Untersuchung der Verbindung zwischen Ca^{2+} und dem JA-Signalweg ist, was besonders in Pflanzen von Bedeutung ist, in denen Mutanten schwer herzustellen sind.

Fluorinierte Jasmonate als Sonde zur Untersuchung des Metabolismus und der Signalwege endogener Verbindungen

Bisher waren keine Hilfsmittel verfügbar um die räumliche und zeitliche Verteilung der Jasmonate in Echtzeit zu beobachten. Daher untersuchte ich die Möglichkeit ein fluoriniertes Jasmonat als Sonde zu benutzen, um metabolische Prozesse zu erforschen. Ich synthetisierte 7F-OPC-8:0 – ein fluoriniertes Analog des JA-Vorläufers OPC-8:0. In *A. thaliana* verhält sich die fluorinierte Verbindung ähnlich zu dem endogenen Oxylipin. Wird sie appliziert, induziert sie sowohl die Akkumulation von Jasmonaten als auch die Expression Jasmonat-regulierter Gene. Unter Verwendung von UHPLC-MS/MS, konnte ich in Blattextrakten Metabolite messen, die durch β-Oxidationen von 7F-OPC-8:0 entstanden sind. Dies ist ein direkter Beweis dafür, dass 7F-OPC-8:0 *in vivo* ähnlich zum endogenen OPC-8:0 metabolisiert wird. Des Weiteren nutzte ich das fluorinierte OPC als Sonde, um dessen Translokation von verletzten Blättern in unverletzte Blätter zu zeigen. Letzteres weist darauf hin, dass der JA-Vorläufer OPC ebenfalls als mobiles Signal zur Induktion systemischer Abwehrreaktionen beitragen kann. Meine Arbeit zeigt das Potential fluorinierter Verbindungen um Jasmonate – und möglicherweise auch weitere Phytohormone und pflanzliche Lipidderivate – in ihrem Metabolismus und Signalweg zu untersuchen.

Perzeption und Inaktivierungsmechanismus des biologisch aktiven Jasmonats JA-Ile. Rationales Design von bioaktiven Jasmonaten

In dieser Studie zeigte ich die Möglichkeit auf, den JA-Ile-Signalweg durch gezielte Modifikationen des Liganden zu manipulieren. Ausgangspunkt war die chemische Struktur des Lactones, das von 12-OH-JA – einem natürlich vorkommenden Jasmonat – abstammt und die Struktur des ternären JA-Ile-Rezeptors Komplexes, der das F-Box-Protein COI1 und einen JAZ-Transkriptionsrepressor enthält. Ich synthetisierte ausgehend von 12-OH-JA-Ile zwei rational designte diastereomere Macrolactone, um die potentielle biologische Aktivität von an C12 modifizierten JA-Ile Derivaten zu untersuchen. Dazu testete ich zunächst, ob diese Lactone die Nikotinproduktion in *N. attenuata* Pflanzen auslösen können. Beide Macrolactone konnten im ähnlichen Maße wie MeJA die Nicotinakkumulation in *N. attenuata* Blättern induzieren. Interessanterweise löste das JA-Ile-Lactone mit der nicht natürlichen Konfiguration die höchste Nikotinakkumulation aus. Diese Ergebnisse veranlassten mich, den JA-Ile-Inaktivierungsmechanismus durch ω-Hydroxylierung in *A. thaliana* zu untersuchen. Mit Hilfe von an C12 modifizierten JA-Ile Derivaten (inklusive der oben genannten Lactone) konnte ich zeigen, dass die Nikotin-induzierenden JA-Ile-Lactone ähnlich wie JA-Ile in *A. thaliana* die Expression von Jasmonat regulierten Genen induzieren können. Das weist darauf hin, dass die freie Carboxylgruppe des JA-Ile für dessen Wirkung nicht essentiell ist. Außerdem werden einige jasmonatregulierte Gene in Abhängigkeit von der Konfiguration des JA-Ile-Lactones unterschiedlich exprimiert. Darüber hinaus, weisen meine Daten darauf hin, dass die verminderte Aktivität des 12-OH-JA-Ile durch eine sterische Hemmung, ausgelöst von der Hydroxylgruppe in der Bindedomäne des JA-Ile-Rezeptors, zustande kommt. Molekülsimulationen zur Förderung weiterer Erkenntnisse über die JA-Ile Wahrnehmung und Inaktivierungsmechanismen sind in Arbeit.

Zusammengefasst liefert meine Forschung den Beweis dafür, dass es möglich ist, den JA-Ile-Signalweg mit Hilfe kleiner Moleküle zu studieren und zu manipulieren. Hiermit präsentiere ich neue Erkenntnisse über den molekularen Mechanismus des JA-Ile-Signalweg von frühen Ereignissen wie der Ca^{2+}-induzierten JA-Ile-Akkumulation, bis hin zu späten Ereignissen wie der Inaktivierung der Jasmonate.

1. General introduction

A sustainable supply of food and energy for the rapidly growing global population has become a huge challenge. A solution to this problem is not obvious, but innovation in plant sciences in order to produce higher crop yields, and plants more resistant to biotic and abiotic stresses could be one of the solutions. In the last years, for instance, significant improvements have been made in molecular biology facilitating the availability of genetically modified crops with high yields.

The lack of motility has forced plants to develop a sophisticated set of defensive strategies. Plants possess complex signaling pathways which can interact among themselves to operate diverse biochemical and physiological responses such as germination, flowering, fruit ripening, photosynthetic regulation, and shoot or root development. Wounding, for example, initiates complex processes designed to protect, repair tissues, and maximize survival of the individuals [1].

In this general introduction I will emphasize particular aspects of plant signaling and extend the literature discussed in the manuscripts that shape my thesis. Furthermore, I will introduce possible research topics which can be examined based on the results obtained in my research.

1.1. Signaling in plants

An external signal may be transmitted to the nucleus of the cell by several mechanisms, one of which is through membrane-localized ion channels. In the presence of a stimulus, ionic characteristics of the cell, as well as the activity of protein kinases change. In the nucleus, the signal can be displayed as a change in the activity of DNA-binding proteins (transcription factors, TF) that specifically interact with and modulate the regulatory regions of genes. In this way, detection of an environmental cue is transmitted and changes in the TF activity regulate the expression of a manifold of genes resulting in developmental reprograming [2].

In most cases, pathways share components at early time points, but differ in later downstream elements, which result in the activation of different target genes. This structure is analogous to the scale-free network architecture (processing multiple signals simultaneously) in computing systems and allows for independent

regulation of genes by the same central signal (equivalent to a hub). Signaling cascades maintain a cross-talk which addresses a particular subset of target genes; they can either increase the response (i.e. synergism) or attenuate the response (i.e. antagonism) [3]. Regulation through repression is a common feature of several signaling pathways (repression is released after signal perception). Positive regulators may be seen as activators of signaling cascades, whilst negative regulators may be responsible for coordinating the exact timed derepression of ongoing responses.

Our understanding of plant signaling pathways has received an extraordinary input from techniques like differential display [4] and DNA chips/microarray [5], and next-generation sequencing [6] which helped to identify new target genes involved in particular processes. As a consequence, the discovery of additional target genes helped to further clarify downstream branches of these signaling pathways.

Everyday complementary genetic, molecular, chemical, and biochemical approaches contribute to the unraveling of the complexity of signal transduction in plants. It is already clear that interactions within a pathway and pathways' crosstalk are extended phenomena. However, the correlation between the signals and activation of the gene expression is yet an exciting field of research [3].

1.1.1. Early events in plant signaling. Ca^{2+}-signaling

The responses of plants to environmental cues occur in a chronological order; they can be classified as early and late events (Figure 1A). Ca^{2+} is an adaptable signaling cation considered as the core of a sophisticated network of signaling pathways. Information from biotic and abiotic factors can be perceived trough changes in Ca^{2+} levels in the cell. Ca^{2+}-signaling has similar characteristics to the bow-tie networks (Figure 1B) [7]. These networks connect inputs and outputs of diverse nature by processing information through a small number of central intermediates. In the cell, Ca^{2+} homeostasis suffers from transient increases in $[Ca^{2+}]_{cyt}$ that emanate from both the external medium and the subcellular compartments. The nature of the external stimulus determines the frequency, amplitude and shape (repetitive oscillations or spiking) of the $[Ca^{2+}]_{cyt}$ burst. It has been postulated that specific changes in $[Ca^{2+}]_{cyt}$ allow this cation to encode

stimulus-specific information. The "Ca^{2+}-hallmark" defines the nature and magnitude of the plant response [7].

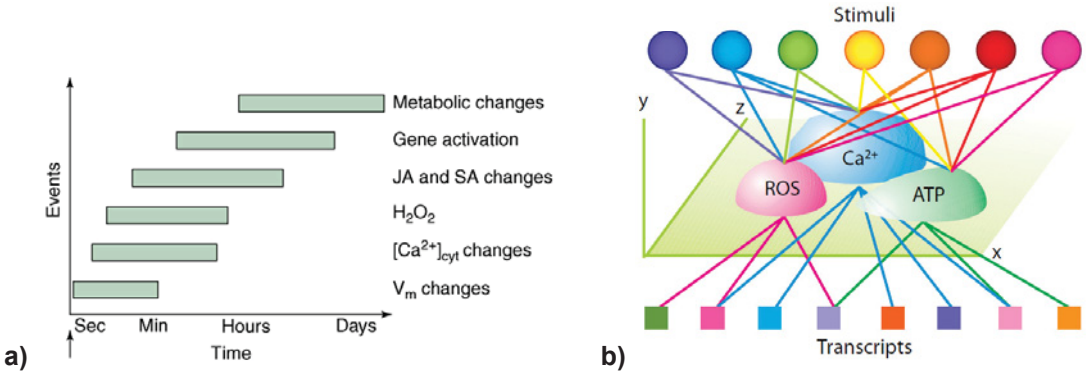


Figure 1. Simplified scheme of signaling events in plants. **a)** Chronology of signaling events in plant-herbivore interaction. The earliest measurable events are voltage changes at the plasma membrane, followed by changes in the $[Ca^{2+}]_{cyt}$ levels and the generation of ROS species. Burst of kinases and phytohormones (e.g., JA, jasmonic acid; SA, salicylic acid) are detectable later. Activation of genes and subsequent metabolic changes are seen at 0.5 to 1 h. Reproduced after [1]. **b)** The bow-tie network architecture concept translated to plant signaling. Diverse inputs (stimuli) regulate diverse outputs (e.g. gene activation). Reproduced after [7]. ROS, reactive oxygen species; ATP, adenosine triphosphate; V_m, voltage of the membrane.

In the Ca^{2+}-signaling pathway, an additional level of regulation and specificity is achieved by Ca^{2+}-binding proteins which can perceive Ca^{2+} fluctuations. These proteins detect increases in $[Ca^{2+}]_{cyt}$ and nuclear free Ca^{2+} that occur prior to or during signal transduction. The number of Ca^{2+}-sensor proteins is large (ca. 3.3% of *A. thaliana* genes are subject to regulation by Ca^{2+}); they differ in Ca^{2+}-binding characteristics, subcellular localizations and downstream signaling interactions. The use of calmodulin (calcium-modulated protein) antagonists like *N*-(6-aminohexyl)-5-chloro-1-naphthelenesulfonamid-hydrochloride and fluphenazine-*N*-2-chloroethane dihydrochloride allowed the identification of 230 Ca^{2+}-responsive genes that were differentially expressed 1 h post treatment [8]. This diversity is thought to provide the necessary tools to decode the information within Ca^{2+} oscillations and its further translation into specific alterations in the function of cell. Ca^{2+}-sensor proteins are classified into two groups: sensor relay proteins (SRL) and sensor responder proteins (SRP). Among SRP are the Ca^{2+}-dependent protein kinases, which combine within a single protein a sensing function (mediated by Ca^{2+}-binding domains) and a response activity (e.g. kinase activity).

SRL may contain multiple Ca^{2+}-binding domains and usually undergo Ca^{2+}-induced conformational changes, but lack an effector domain [7]. Therefore, SRLs must interact with other proteins in order to transmit the signal.

There is not much understanding of the way in which Ca^{2+} signals are transduced to the transcription machinery, but Ca^{2+}-calmodulin-binding transcription activator proteins (CAMTA) seem to be involved in this process. There are six members of the CAMTA family in *A. thaliana* and various stimuli including cold, salinity and phytohormones influence rapidly and transiently their gene expression [9]. Calmodulin-like proteins (CML) are also involved in Ca^{2+} signal transduction. Analysis of *cml42* mutants revealed that they are more resistant to herbivory than wild type (WT) plants. It was demonstrated that this gene regulates plant defenses negatively, as caterpillars gained less weight feeding on the mutant. High content of aliphatic glucosinolates and transcript accumulation of the *VSP2* and *Thi2.1* genes was reported for the *cml42* mutant after herbivory. Furthermore, root growth inhibition and Ca^{2+}-induced elevation of JAs were more noticeable in *cml42* plants compared to WT indicating JAs hypersensitivity [10]. On the contrary, the *cml37* mutant (same CML family) showed no changes in the content of herbivory-induced secondary metabolites, while the accumulation of JAs was significantly reduced due to low expression of *JAR1* (jasmonic acid-amido synthetase) with the corresponding deficient enzyme activity. Contrary to *cml42*, *S. littoralis* larvae gained more weight feeding on *cml37* plants than on WT plants, suggesting that *CML37* is a positive plant defense regulator [11].

Calcium-dependent protein kinases (CPKs) classify in the SRP subgroup of Ca^{2+}-sensor proteins and their involvement on JA signaling have been suggested. In *N. attenuata* plants, for instance, silencing of *CPK4* and *CPK5* genes led to the higher accumulation of JA and defense metabolites which potentiated the plant resistance to attack from *Manduca sexta* larvae [12]. A regulation of early steps of the JA biosynthetic pathway was suggested for *CPK4* and *CPK5* genes, more likely acting on the AOS and AOC enzymes [13].

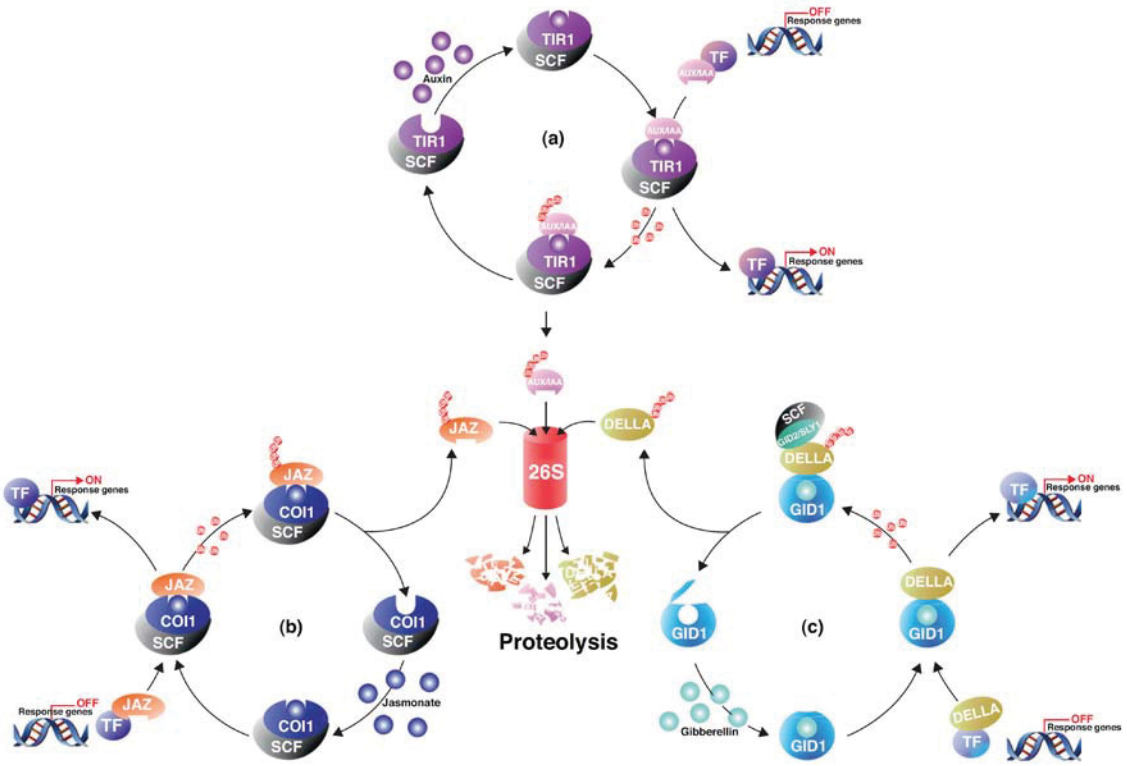
The two pore channel 1 gene (*TPC1*) encodes a non-selective cation channel (Ca^{2+}-permeant) in *A. thaliana*. A missense mutation in the putative voltage sensor

of this gene causes faster time-dependent conductivity and activation of the mutated channel at lower membrane potentials than the channel in WT plants. This was associated with a misregulated activity of 13-lipoxygenase (13-LOX) at the beginning of the JA biosynthetic pathway [14]. Taken all together, the above mentioned facts provide strong evidence of the close relationship between the Ca^{2+} and the JA signaling pathways.

1.1.2. Late events in plant signaling. Signaling by small molecules

Phytohormones appear at later time points compared to Ca^{2+} in the signal transduction pathway (Figure 1A). Nowadays it is accepted that a complex crosstalk and induced hormonal changes modulate development and resistance in plants [15]. Several similarities in signaling mechanisms have been revealed between different hormones. One of these similarities is that gene induction is often achieved by derepression (Scheme 1). Positive and negative regulators of hormone signaling pathways are common targets of the different hormones [16].

There are several phytohormones; each of them having distinct and sometimes overlapping roles in the regulation of the plant development and defense. SA is a hormone mediating resistance against biotrophic pathogens, whereas resistance against necrotrophic pathogens is mediated by a combination of JA and ethylene (ET). These two processes are generally antagonistic. Elevated biotroph resistance is often correlated with increased necrotroph susceptibility and vice versa [17]. Gibberellic acid (GA) controls degradation of the DELLA (negative regulators of gibberellin) proteins, and therefore, the accumulation of ROS, SA and depletion of JA [18]. Brassinosteroids enhance biotrophic resistance and also mediate abiotic stress responses [19]. Cytokinins (CKs) magnify the SA responses through the NPR1 (non-expressor of pathogenesis related 1), and consequently, enhance resistance against biotrophs [20]. Auxin signaling suppresses SA biosynthesis and signaling [21], while SA treatment reduces the auxin-dependent degradation of AUX-IAAs (auxin/indole-3-acetic acid proteins) that negatively regulate auxin signaling [21]. For comprehensive reviews on phytohormones crosstalk and signaling mechanisms see references [15,22,23].



Scheme 1: Simplified scheme of the auxin **(a)**, JA **(b)** and GA **(c)** signaling pathways. Activation is mediated by the degradation of negative regulators AUX-IAA, JAZ, and DELLA respectively. The negative regulators bind to, and inactivate the positive regulators of each pathway, the TFs ARF, MYC2/3/4, and PIF3/4 respectively. Signaling generally involves changes in transcriptional regulation. The central role of the 26S proteasome is depicted. A second layer of repression (not shown) is added in **(a)** and **(b)** by the presence of the general repressor TOPLESS (TPL) that stabilizes the complexes in their inactive state. Scheme reproduced after [22].

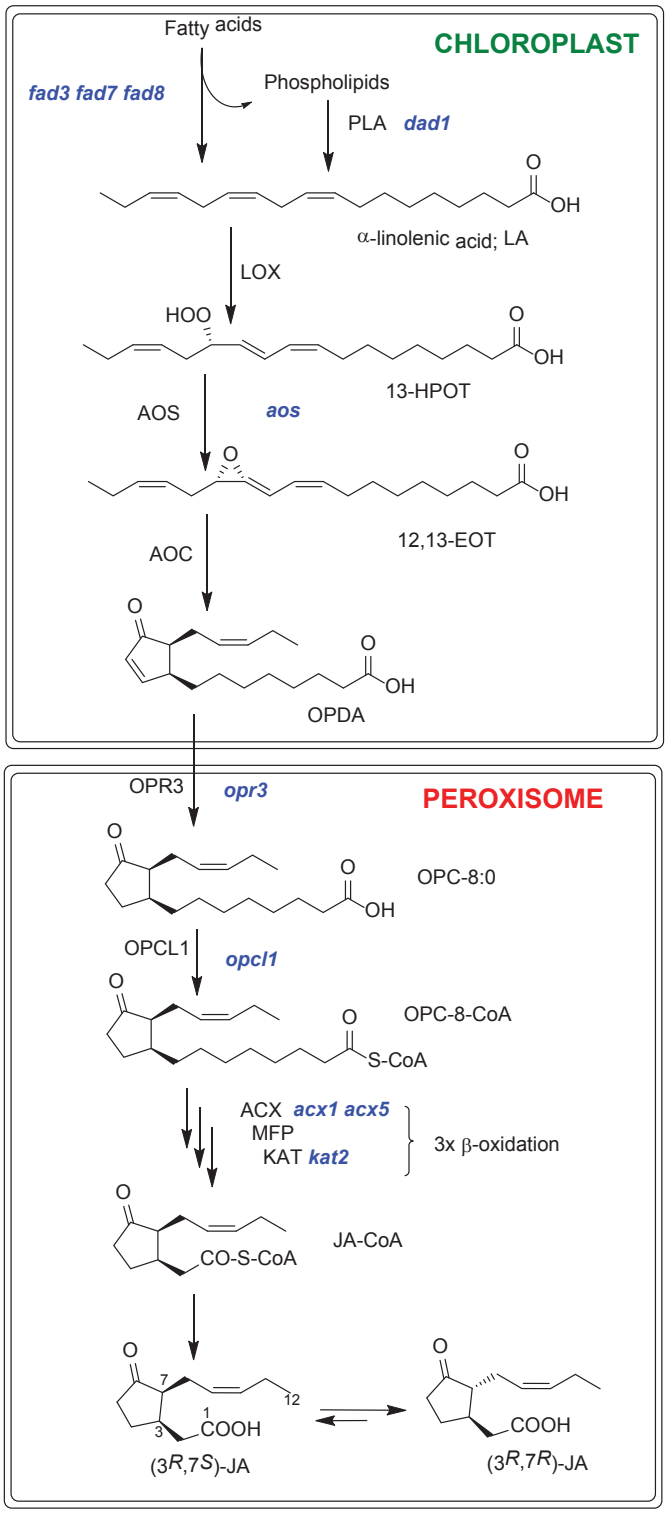
Among phytohormones, JAs act as master regulators of plant defenses and this discovery was a key advance in better understanding plant-stress biology. These phytohormones are particularly interesting because of their dual role in inhibiting growth and promoting defense and reproduction. JA has been isolated from various plant species. Its biological role as a growth inhibitor has been extensively studied [24-26]. Several mutants in the JA biosynthetic pathway helped to elucidate the JA biosynthesis mechanism [27]. Some of the mutations resulted in male sterility, e.g. *A. thaliana* flowers of JA-deficient or JA-insensitive mutants have shorter anther filaments and defects in pollen development [28,29]. JAs are well established “wound hormones” playing an important role in defense against biotic stresses [30,31]. Furthermore, JAs are also involved in plant responses to abiotic stresses including responses to UV light and salt stress [32,33].

JAs are of great significance not only in agricultural research, but also in medicine, where several JAs have been studied as antitumor compounds recently [34,35]. It is evident that understanding the action mechanism of this compound family is of great interest. In the following sections I will cover some key aspects of the metabolism and physiology of these plant metabolites.

1.1.2.1. JA biosynthesis

JAs are lipid-derived molecules participating in many plant development processes, biotic and abiotic stress responses [27,28,36]. They share a high degree of structural and functional similarities with prostaglandins found in animals. Fatty acids are the precursors of both types of compounds and their biosynthesis is induced in response to stress [26,36]. JA is the basic constituent of the JAs family. Plant defenses are severely compromised when JA biosynthesis or perception is deficient. Experiments using the *aos* (allene oxide synthase) LoF ─ loss-of-function ─ mutant suggested that ca. 95% of wound-stimulated changes in protein levels are JA-dependent [37].

The JA biosynthetic pathway is well understood and most of the enzymes participating in it are well characterized by several methods (Scheme 2) [27,38,39]. It starts in the plastid with the release of the unsaturated fatty acids α-linolenic acid (LA) and hexadecatrienoic acid (HTA) from the plastidic glycerolipids. This was demonstrated by using the *A. thaliana* triple mutant *fad3/fad7/fad8* which is impaired in both fatty acids desaturation and JA biosynthesis [40]. Lipases DAD1 (defective in anther dehiscence 1) and its paralog DGL (dongle) are involved in the release of LA [41]. 13-LOX (13-lipoxygenase) is capable of oxidizing LA to 13-hydroperoxy linolenic acid (13-HPOT) which can be metabolized to different classes of oxylipins [36]. The conversion of 13-HPOT into 12,13-epoxyoctadecatrienoic acid (12,13-EOT) by the AOS is the main transformation of 13-HPOT. The *AOS* gene has only one copy in *A. thaliana* and it has been clearly shown that the *aos*-LoF mutant is deficient in JA [29]. The AOC (allene oxide cyclase) acts on 12,13-EOT to produce *cis*-(+)-12-oxo-phytodienoic acid (OPDA) stereospecifically. OPDA is the first compound having the cyclopentanone ring and remarkable JA-related bioactivity [42]. The same set of enzymes convert HTA into dinor-OPDA which is two methylenes shorter than



Scheme 2: Simplified scheme of the JA biosynthesis (LOX pathway). LA (18:3) is released from the glycerolipids by phospholipases in the chloroplast. The action of LOX results in the conversion of LA into the hydroperoxide 13-HPOT. Following this step, AOS transforms 13-HPOT into the unstable allene oxide 12,13-EOT, which is further converted to OPDA by the AOC enzyme. OPDA is translocated to the peroxisomes and there reduced to OPC-8:0 by OPR3. OPC-8:0 is activated to the corresponding CoA ester by OPCL1 (OPC-8:0-CoA ligase 1). This is the entry-point to three β-oxidations carried out by ACX (acyl-CoA oxidase), MFP (multifunctional protein) and KAT (L-3-ketoacyl CoA thiolase). Jasmonoyl-CoA, is likely cleaved by a thioesterase to produce (3*R*,7*S*)-JA, which can epimerizes to the more stable (3*R*,7*R*)-JA. *A. thaliana* mutants that have been important for elucidating the JA biosynthetic pathway are indicated in blue. Scheme modified after reference [39].

OPDA at the carboxyl side chain. In *A. thaliana*, dinor-OPDA and OPDA are stored by esterification to glycerolipids and can be released in their free form when the plant faces different challenges such as virulence proteins [43]. Further conversion of OPDA implies its translocation from the chloroplasts to the peroxisomes. Afterwards, OPR3 (OPDA reductase 3) catalyzes the reduction of OPDA to 8-

((1*S*,2*S*)-3-oxo-2-((*Z*)-pent-2-en-1-yl)cyclopentyl)octanoic acid (OPC-8:0). OPR3 seems to be the only isoform found in the *A. thaliana* peroxisomes as the *opr3*-LoF mutant is JA-deficient [28]. Three rounds of β-oxidation are required for shortening the carboxyl side chain of OPC-8:0. This is performed by the enzymes ACX, MFP, and KAT acting in consecutive order. The final product of the β-oxidations, jasmonoyl-CoA, is cleaved by a putative thioesterase producing (3*R*,7*S*)-JA that can epimerize to the more stable isomer (3*R*,7*R*)-JA.

1.1.2.2. Systemic transport of JAs and precursors

Little is known about the systemic translocation of JAs and their role in systemic resistance. Similarly poor is the knowledge concerning JAs precursors like OPDA and OPC-8:0. Detailed literature overview about this topic is given in the chapter 5 (Article II) of my thesis. Therefore, in this general introduction, I limit my comments to the potential applications of the compound prepared in that work. I briefly discuss the potential of fluorinated JAs in the study of the translocation of these compounds in real time by using positron emission tomography (PET).

1.1.2.2.1. PET to study transport phenomena in plants

The yield of crops is determined not only by the genotype, but also by the growth environment of the plant. Therefore, phenomics has become a powerful tool in plant research and innovation [44]. Most phenotyping systems are based on visible light imaging techniques, although x-ray computed tomography (CT) and magnetic resonance imaging (MRI) have also been adapted to image the inner structures of plants or their roots in the soil [45]. Apart from the previously mentioned techniques, other non-destructive imaging methods (e.g. PET) that could reveal physiological information are of great value for the future plant phenomics [45,46].

PET is an imaging technique that produces 3D images of functional processes in an organism [47]. It is based on the detection of pairs of oppositely-directed gamma rays emitted by a positron-emitting radionuclide, called tracer, which is chemically attached to the molecule of biological interest. Computer analysis allows the construction of concentration-dependent 3D images of the tracer within the organism. This technique is widely used in medicine, often coupled to CT

(PET-CT scanners). Isotopes commonly used in PET should have a short half-life, clear β^+-decay, and the quantity of γ-photons having energy other than 511 keV should not exceed 0.1 %. The four most commonly use PET-isotopes are carbon-11 (^{11}C), nitrogen-13 (^{13}N), oxygen-15 (^{15}O), and fluorine-18 (^{18}F); being ^{18}F the most widely employed. In medicine, for example, ^{18}F-fluorodeoxyglucose, a fluorinated analogue of glucose, is a biologically active molecule chosen for ca. 90% of the PET scans. However, other radiotracers may be employed depending on the peculiarity of the study [48].

PET has also been used for plant research. First studies on photo-assimilate transport in plants using ^{11}C labeled CO_2 ($[^{11}C]CO_2$) were carried out in the early 60s [49]. In 1988 McKay et al. obtained dynamic images of long distance nutrient translocation in plants. In this study positron emitting ^{18}F was fed in solution to excised stems in a large-aperture PET detector. The resolution obtained in time and space was of 0.5 min and 4 mm respectively [50]. The major difficulties these authors found were i) the limited availability of scanning systems and ii) the lack of a suitable radiotracer. However, nowadays most PET scanners employed in plant imaging research are based on high resolution PET detectors, commonly used for small animal PET. In Germany, for instance, researchers from the Central Institute for Electronics of the Research Center Jülich have developed a 3D imaging system for plant studies with 1.3 mm of spatial resolution (Figure 2) [51].

^{18}F labeling offers the possibility of measuring long-term phenomena due to its prolonged half-life time (~110 min radioactive half-life). Besides, many synthetic procedures were developed recently for the ^{18}F-labeling of diverse molecules: Ligands to study vasoactive intestinal peptide receptors [52], ^{18}F-“click” radiolabeling [53], solid-phase radiolabeling using 4-[^{18}F]-fluorobenzoic and 2-[^{18}F]-fluoropropionic acids [54] and ^{18}F labeling by isotopic exchange ($^{18}F/^{19}F$) [55] are just some examples.

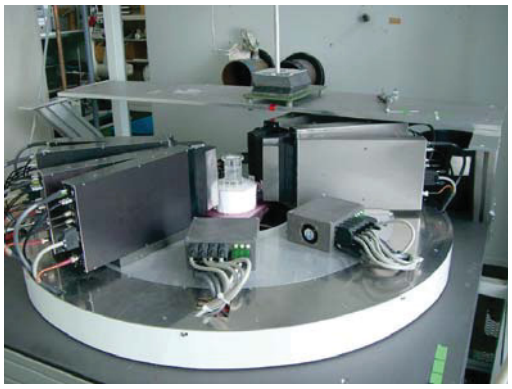


Figure 2: The PET system developed in the Research Center Jülich. It has eight detector cassettes arranged in two opposed detector blocks of four cassettes each. It offers a resolution of ca. 1.3 mm. Reproduced after reference [51].

^{18}F-tracers have not been employed in plant research as much as in medicine, however, a few studies used ^{18}F as a proxy for tracing the dynamics of water transport. This was possible due to the capability of fluorine to form hydrogen bonds with water molecules [50,56]. In the near future, the ^{18}F-labeling of small molecules with biological relevance (phytohormones, secondary metabolites, etc.) should facilitate the *in vivo* exploration of dynamic long-term processes. Obviously, the use of ^{18}F-labeled tracers in plant biology can benefit from techniques and methods which are already well stablished in medical research. My work on fluorinated jasmonates (see chapter 5, article II) should pave the road to study the translocations of JAs in real time by using PET.

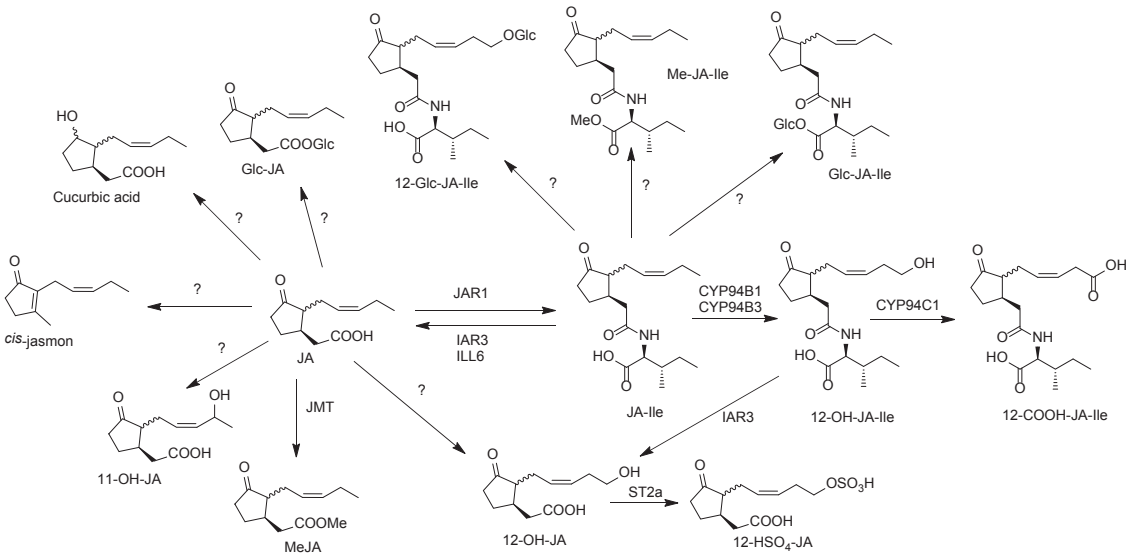
1.1.2.3. Metabolic conversions of JA

A manifold of JA-derived metabolites have been detected in various plant species. This constitutes direct evidence that JAs are the target of several enzymes [38]. Among these metabolites are amino acid (Ile, Leu, Val, Tyr, Trp) conjugates of JA, where *N*-jasmonoyl-L-isoleucine (JA-Ile) is the most important one [57]. Methyl and glucose esters, together with hydroxylated derivatives are also commonly occurring JA derivatives. Scheme 3 shows some of the JAs previously reported to occur in nature and enzymes performing the transformations.

1.1.2.4. JA signaling

When a plant is challenged by external cues, e.g. herbivory, a burst of JA/JA-Ile is produced. Afterwards, the bioactive endogenous jasmonate JA-Ile [57] binds to the receptor complex SCF^{COI1} (a Cullin-RING E3 type ligase) [58,59], which targets the JAZ (jasmonate-ZIM-domain) transcript repressors for degradation *via* the 26S

proteasome pathway (see Scheme 1b). This allows TFs (e.g. MYC2/3/4) to activate JRG expression [60].



Scheme 3: Simplified scheme of metabolic conversions of JAs. Conjugation of JA with isoleucine by JAR1 generates the natural bioactive jasmonate JA-Ile, which can be converted later to partially inactive 12-OH-JA-Ile by two JA-Ile hydroxylases (CYP94B3 and CYP94B1). In a successive oxidation step 12-OH-JA-Ile is transformed into the inactive 12-COOH-JA-Ile by CYP94C1. Hydroxylation of JA yields 12-OH-JA, which can be sulfated to 12-HSO_4-JA by ST2a (sulfotransferase). JA can also be hydroxylated to 11-OH-JA or methylated at the carboxyl group by JMT (JA methyltransferase). A question mark indicates enzymes yet unknown. Note that only a selection of JA metabolites is shown and they are mostly present as mixture of diastereoisomers indicated by the wavy bond. Adapted from reference [39].

The family of the JAZ proteins is form by at least 12 members in *A. thaliana*. JAZ proteins repress JA-Ile signaling [61,62]. Their mechanism of action is very similar to the one described for AUX-IAA proteins repressing auxin signaling (Scheme 1a). JAZs can interact (homo- and heterodimerize) *via* the conserved TIFY domain they possess [63]. Another conserved domain among JAZs, the C-terminal jasmonate associated domain (Jas), facilitates the binding to TFs for repression and also the interaction with the receptor F-box protein COI1 (coronatine insensitive 1) [64]. With the degradation of the JAZs, derepression of TFs (MYC2/3/4) facilitates activation of JRGs [65]. MYC2 is probably the most important TF involved in the JA-dependent transcriptional changes in *A. thaliana* [26]. Moreover, COI1 mediates the majority of JA-related responses [29,58,66], although some COI1-independent pathways have been reported [28].

Recently, the crystal structure of the JA-Ile receptor complex has been elucidated in *A. thaliana* [67,68]. It has been shown that high-affinity assembly of the SCF^{COI1}/JA-Ile/JAZ complex requires also inositol pentakisphosphate as co-factor. JA-Ile interacts with both the JAZ and COI1 [67,68]. The fine-tuning of the JA-Ile perception mechanism seems to be assisted by the homo- and heterodimerization capacity among the different JAZs [63] and the JAZs with other general repressors [69]. Moreover, JAZ proteins attenuate JA-Ile signaling by forming SCF^{COI1}-JAZ complexes where the JAZ (e.g. JAZ8) is stable to ubiquitination and degradation [70].

The amplitude and duration of JA-related responses is controlled mainly by JA-Ile, but when the signal molecule is no longer needed, enzymes involved in JA-Ile metabolism and turnover play a primary role. Although little is known about these processes, some important components were revealed recently. On the one hand, it has been shown that two members of the ILR1-LIKE auxin amidohydrolase family (IAR3 and ILL6) contribute to JA-Ile turnover during the wound response in *A. thaliana* leaves. These two enzymes cleave JA-Ile with the subsequent release of L-Ile and JA. Besides, there is evidence that they can also split 12-OH-JA-Ile to produce the corresponding 12-OH-JA [71]. On the other hand, it was shown that ER-localized cytochromes P450 (CYP94B1/B3/C1) sequentially oxidize JA-Ile to 12-OH-, 12-CHO- and 12-COOH-JA-Ile derivatives (Scheme 3) [72,73]. Analysis of *cyp94b1*-LoF and overexpressing plants revealed that the coordinated action of CYP94B1 and CYP94B3 controls the majority of the ω-hydroxylation of JA-Ile in wounded leaves. Furthermore, the JA profile of a double LoF-mutant (*cyp94b3* and *ill6*) suggested that the turnover of JA-Ile is the result of the collaborative action of the oxidative and amidolytic pathways [72].

1.1.1.1. Structure-activity relationships of JAs

The high number of JAs found in plants, and the differences in their bioactivity raise one important question: How are the structural differences connected to the biological activities of these molecules? Since Ueda and Kato reported on the JAs-induced plant growth inhibition [25] the study of structure-activity relationships (SAR) of JAs became a very interesting field of research. At the beginning, SAR studies were carried out by the direct application of the molecule to the system of

interest, followed by the examination of various biological responses including tuber formation, root growth inhibition, tendril coiling, alkaloid production, and the expression of JRGs [74-76]. The data obtained by following this methodology provided only initial hints on SAR of JAs, mainly because the results were dependent on several factors, such as: the concentration and polarity of the compound (membrane permeability), duration of the treatment, and the possibility of *in vivo*-conversion to a more or a less active derivative.

Coronatine is a structural and functional mimic of JA-Ile. This phytotoxin is produced by several strains of *Pseudomonas syringae* and has a higher biological activity than JA-Ile [77]. Synthetic rationally-designed analogues of Coronatine (e.g. Coronalon and other 6-substituted-4-oxo-indanoyl-isoleucine conjugates) showed particular high bioactivity as well [78]. The defined stereochemistry of synthetic JAs, in combination with the differential bioactivity of particular JA stereoisomers revealed some structural requirements for JAs bioactivity. Some of these requirements are: presence of a cyclopentanone ring [28], stereochemistry at C3 and C7 [57], the pentenyl side chain on C7 [38,79], and presence of a –C-COOH or –C-$COOCH_3$ group on C3 for amino acid conjugation [38].

It has been demonstrated that ω-hydroxylation of JA leads to partial suppression of JA signaling [72,80,81]. Furthermore, wounding-induced accumulation of 12-glucoside-JA and other 12-modified-JA derivatives has been observed, which indicates a physiological role of this type of JA-metabolites in switching off JA signaling [82]. However, some of these compounds, 'less active' in JA signaling, are deeply involved in other biological functions, such as leaf-closing movement [83]. Interestingly, most of the SAR studies mentioned above were carried out before two very important discoveries were made. The first one, is the discovery of the biologically active jasmonate [57,84], namely JA-Ile; and the second one is the crystal structure elucidation of the JA-Ile receptor complex [67]. In my thesis I use this valuable information to further study the SAR of jasmonates with the help of rationally-designed JA-Ile derivatives (see chapters 6 and 7, article III and unpublished results respectively).

2. Aims and scope of the thesis

In the introduction it was outlined that phytohormones are of extraordinary importance for plants. Among plant hormones, JAs play an important role in several processes such as growth, photosynthesis, cell death, leaf senescence, and reproductive development. Thus, it is of great interest to understand mechanistic details on how exactly JAs are implicated in plant signaling and metabolism.

The general aim of my dissertation is to study different aspects of JA signaling in plants with the help of JAs and JAs-derivatives synthesized chemically. Herein, I intend to highlight the potential of rational synthetic approaches to facilitate and complement the study of the JA signaling pathway. In particular, I discuss the following features:

- **Early events in JA signaling.**
  - ✓ **The relationship between Ca^{2+} signaling and JA-signaling.** I employ synthetic JA-Ile and 12-OH-JA-Ile to study the connection between $[Ca^{2+}]_{cyt}$ elevation and JA-Ile accumulation and signaling. Results of this study are described in the chapter 4 (article I).
- **Late events in JA signaling. Translocation and perception of JAs.**
  - ✓ **The role of JAs precursors in JA signaling.** I aim to synthesize a fluorinated analog of the JA precursor OPC-8:0 to study the metabolism and translocation of the synthetic compound. The results of this work are presented in chapter 5 (article II).
  - ✓ **Rational design of new bioactive JAs.** This study is dedicated to the exploration of the biological activity of two rationally designed JA-Ile analogues. Outcomes are described in chapter 6 (article III).
  - ✓ **Inactivation of the active jasmonate JA-Ile.** A SAR study is carried out to investigate the "switch-off" mechanism of JA-Ile upon ω-hydroxylation. This research is described in chapter 7 as unpublished results.

To sum up, within this thesis, I cover single aspects of the JAs signaling ranging from early events in plant defenses (activating JA biosynthesis) to the turnover of the bioactive jasmonate JA-Ile.

3. Thesis outline – List of articles and unpublished results. Authors' contribution

3.1. Article I: Neomycin inhibition of (+)-7-*iso*-jasmonoyl-L-isoleucine accumulation and signaling

The action of the antibiotic neomycin on the oral secretion (OS) induced Ca^{+2} elevation and levels of jasmonates in *A. thaliana* plants is described. It is shown that neomycin selectively blocks the OS-induced Ca^{+2} elevation and reduces the level of the bioactive jasmonate JA-Ile, in contrast to the level of its precursor JA. Consequently, neomycin treatment affects the downstream expression of JA-Ile responsive genes *VSP2* and *LOX2*. The neomycin-dependent reduction of JA-Ile is partially due to the increased expression of CYP94B3 responsible for the conversion of JA-Ile into inactive 12-OH-JA-Ile. This suggests that Ca^{+2} burst specifically controls JA-Ile accumulation and signaling. The work offers new insights into the role of Ca^{+2} in defenses against insect herbivory and its direct link with jasmonate signaling.

Publikation	Neomycin inhibition of (+)-7-iso-jasmonoyl-L-isoleucine accumulation and signaling. Jyothilakshmi Vadassery, Michael Reichelt, Guillermo H. Jimenez-Aleman, Wilhelm Boland and Axel Mithöfer. *Journal of Chemical Ecology*, **2014**, 40(7), 676-86. DOI: 10.1007/s10886-014-0448-7				
Beteiligt an	J. Vadassery	M. Reichelt	G.H. Jimenez-Aleman	W. Boland	A. Mithöfer
Konzeption des Forschungsansatzes	X				X
Planung der Untersuchungen	X		X		X
Datenerhebung	X	X	X		
Datenanalyse und -interpretation	X	X	X	X	
Schreiben des Manuskripts	X				X
Vorschlag Anrechnung Publikationsäquivalente	0,5 (contribution ~ 25%)				

3.2. Article II: Synthesis, metabolism and systemic transport of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0

In this work, it is investigated whether fluorinated jasmonates could mimic the action of the endogenous compounds and therefore, be employed as probes or tracers to study plants metabolic processes. I carried out the synthesis of (*Z*)-7-fluoro-8-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)octanoic acid (7F-OPC-8:0), a fluorinated analogue of the JA-precursor OPC-8:0, and studied its metabolism and systemic translocation. The fluorinated compound induces the transcription of marker JRG and the accumulation of jasmonates similarly to other endogenous JAs after application to *A. thaliana* plants. An UHPLC-MS/MS method was developed to identify 7F-OPC-8:0 and derivatives showing that this compound is metabolized *in vivo* similarly to the endogenous OPC-8:0. Furthermore, 7F-OPC-8:0 was successfully employed as a probe to study its translocation to systemic undamaged leaves when applied to wounded local leaves. The results suggested that OPC-8:0 ─ and maybe other oxylipins ─ may contribute to the mobile signal which triggers systemic defense responses in the plant. The potential of fluorinated oxylipins to study the mode of action of lipid-derived molecules *in planta* is highlighted. Availability of 7F-OPC-8:0 will enable the replacement of the fluorine atom by the respective radioactive isotope to study transport phenomena in real-time using PET.

Publikation	Synthesis, metabolism and systemic transport of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0. Guillermo H. Jimenez-Aleman, Sandra S. Scholz, Monika Heyer, Michael Reichelt, Axel Mithöfer and Wilhelm Boland. *Biochimica Et Biophysica Acta-Molecular And Cell Biology Of Lipids*, **2015**, 1851, 1545-1553. DOI: 10.1016/j.bbalip.2015.09.002					
Beteiligt an	G.H. Jimenez-Aleman	S.S. Scholz	M. Heyer	M. Reichelt	W. Boland	A. Mithöfer
Konzeption des Forschungsansatzes	X	X			X	X
Planung der Untersuchungen	X	X	X		X	X
Datenerhebung	X	X	X	X		
Datenanalyse und -interpretation	X	X	X		X	X
Schreiben des Manuskripts	X					
Vorschlag Anrechnung Publikationsäquivalente	1,0 (contribution ~ 40%)					

3.3. Article III: Synthesis, structural characterization and biological activity of two diastereomeric JA-Ile macrolactones

Inspired by the chemical structure of jasmine ketolactone, a naturally occurring 10-membered macrolactone, and in order to further explore potential biological activities of 12-modified JA-Ile derivatives, I synthesized two diastereomeric macrolactones derived from 12-OH-JA-Ile. The biological activity of these compounds was tested for their ability to elicit nicotine production, a well-known jasmonate dependent secondary metabolite. Both macrolactones showed strong biological activity, inducing nicotine accumulation to a similar extent as MeJA does in *Nicotiana attenuata* leaves. Surprisingly, the highest nicotine contents were found in plants treated with the JA-Ile-lactone having the (3*S*,7*S*) configuration at the cyclopentanone ring, which is not present in natural JAs. These synthetic macrolactones are valuable standards to explore SAR of JA-Ile signaling and whether such a lactone exists in nature. All co-authors were involved in the discussion of results, read and approved the final version of the manuscript.

Publikation	Synthesis, structural characterization and biological activity of two diastereomeric JA-Ile macrolactones. Guillermo H. Jimenez-Aleman, Ricardo A. R. Machado, Helmar Görls, Ian T. Baldwin, and Wilhelm Boland. *Organic and Biomolecular Chemistry*, **2015**, 13, 5885. DOI: 10.1039/c5ob00362h				
Beteiligt an	G.H. Jimenez-Aleman	R.A.R. Machado	H. Görls	I.T. Baldwin	W. Boland
Konzeption des Forschungsansatzes	X	X			X
Planung der Untersuchungen	X	X			X
Datenerhebung	X	X	X		
Datenanalyse und -interpretation	X	X	X	X	X
Schreiben des Manuskripts	X				
Vorschlag Anrechnung Publikationsäquivalente	1,0 (contribution ~ 70%)				

3.4. Unpublished results: Steric hindrance as key factor for inactivation of JA-Ile after ω-hydroxylation

A rationally designed synthetic approach to investigate the switch-off mechanism of JA-Ile upon hydroxylation at C12 in *A. thaliana* is described. In this work SAR studies were carried out with the help of synthetic analogues of JA-Ile. It is shown that i) inactivation of JA-Ile upon ω-hydroxylation to occurs due to steric hindrance caused by the introduced hydroxyl group at C12, ii) the JA-Ile-lactone synthesized from inactive 12-OH-JA-Ile is capable of activating a subset of JRGs similarly to the active JA-Ile, and iii) a free carboxyl group is not necessary for JA-Ile to interact with its receptor, while an ester group in the L-Ile moiety is sufficient. Modelling calculations, which give further insights into the system, are in progress. This study has not only revealed new aspects of the JA-Ile signaling mechanism, but also provided evidence of the possibility of manipulating the JA-Ile signaling pathway by tailored modifications to the natural ligand. All co-authors were involved in the discussion of results, read and approved the final version of the manuscript.

Publikation	Inactivation of JA-Ile after ω-hydroxylation: Steric hindrance may be the key. Guillermo H. Jimenez-Aleman, Jyothilakshmi Vadassery, Wolfgang Brandt, Axel Mithöfer and Wilhelm Boland. (in preparation)				
Beteiligt an	G.H. Jimenez-Aleman	J. Vadassery	W. Brandt	A. Mithöfer	W. Boland
Konzeption des Forschungsansatzes	X				X
Planung der Untersuchungen	X	X			X
Datenerhebung	X	X	X		
Datenanalyse und -interpretation	X	X	X	X	X
Schreiben des Manuskripts	X				
Vorschlag Anrechnung Publikationsäquivalente	1,0 (contribution ~ 65%)				

3.5. Article IV: Literature Review: Methyltrioxorhenium (VII). Second Update

This article was published in an e-book dedicated to the state-of-the-art in enantiospecific and diastereospecific catalytic oxidation of organic substrates. It contains synthetic information for the versatile reagent Methyltrioxorhenium (VII). Reactions covered in the publication include epoxidation and metathesis of alkenes, deoxygenation of diols and epoxides, and oxidation of nitrogen-containing compounds. The literature compiled in this manuscript contributed to enrich my knowledge of organic synthesis, facilitating in that way the synthetic work carried out throughout my PhD.

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Beteiligt an	G.H. Jimenez-Aleman	W. Boland
Konzeption des Forschungsansatzes		
Planung der Untersuchungen		
Datenerhebung		
Datenanalyse und -interpretation	X	X
Schreiben des Manuskripts	X	
Vorschlag Anrechnung Publikationsäquivalente	0,5 (contribution ~ 90%)	

4. Article I

Neomycin inhibition of (+)-7-*iso*-jasmonoyl-L-isoleucine accumulation and signaling

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Neomycin Inhibition of (+)-7-*Iso*-Jasmonoyl-L-Isoleucine Accumulation and Signaling

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Abstract The majority of plant defenses against insect herbivores are coordinated by jasmonate (jasmonic acid, JA; (+)-7-*iso*-jasmonoyl-L-isoleucine, JA-Ile)-dependent signaling cascades. Insect feeding and mimicking herbivory by application of oral secretions (OS) from the insect induced both cytosolic Ca^{2+} and jasmonate-phytohormone elevation in plants. Here it is shown that in *Arabidopsis thaliana* upon treatment with OS from lepidopteran *Spodoptera littoralis* larvae, the antibiotic neomycin selectively blocked the accumulation of OS-induced Ca^{2+} elevation and level of the bioactive JA-Ile, in contrast to JA level. Furthermore, neomycin treatment affected the downstream expression of JA-Ile-responsive genes, *VSP2* and *LOX2,* in Arabidopsis. The neomycin-dependent reduced JA-Ile level is partially due to increased *CYP94B3* expression and subsequent JA-Ile turn-over to12-hydroxy-JA-Ile. It is neither due to the inhibition of the enzymatic conjugation process nor to substrate availability. Thus, blocking Ca^{2+} elevation specifically controls JA-Ile accumulation and signaling, offering an insight into role of calcium in defense against insect herbivory.

Keywords Herbivory · Jasmonates · Neomycin · Plant defense · *Spodoptera littoralis*

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Introduction

Plants, when attacked by herbivorous insects are able to recognize the attack and react accordingly with the induction of various direct and indirect defense responses (Mithöfer et al. 2009; Mithöfer and Boland 2012). A combination of mechanical wounding during the feeding process and chemical signaling compounds derived from the herbivore are responsible for final elicitation of a specific defense response (Mithöfer and Boland 2008). Upon recognition of herbivory, an intracellular signaling cascade is necessary for the activation of defensive reactions. Intracellular Ca^{2+} concentration changes as well as phytohormones, in particular the jasmonate pathway, play a dominant role in the signaling process (Maffei et al. 2007). For example, about 95 % of protein re-patterning near wound sites is jasmonate dependent (Gfeller et al. 2011). Jasmonates are oxylipin phytohormones derived from linolenic acid and are involved in plant development and defense responses (Wastenack 2007). The key compound among the jasmonates, jasmonic acid (JA), is the direct precursor of the bioactive form; the latter was identified as (3*R*,7*S*)-*N*-jasmonoyl-L-isoleucine (JA-Ile; Fonseca et al. 2009). In the interaction between *Arabidopsis thaliana* and larvae of the generalist insect herbivore *Spodoptera littoralis*, elicitors in the caterpillars' oral secretions (OS) induce cytosolic [Ca^{2+}] elevation, prolonged JA and transient JA-Ile burst in treated Arabidopsis leaves, within a few minutes after their application (Vadassery et al. 2012a). *Spodoptera littoralis* OS is a mixture of secretions from salivary glands and gut reflux, referred to as regurgitate, that also contains plant-derived and microbial compounds. By following the insect-catalyzed conversion of *cis*-OPDA (12-oxo-phytodienoic acid, a biosynthetic precursor of JA) into *iso*-OPDA, a continuous regurgitation of insects was demonstrated while feeding on Arabidopsis leaves (Vadassery et al. 2012b). Unfortunately, the active factor in OS that is responsible for the above

mentioned responses, *e.g.*, cytosolic [Ca^{2+}] elevation, JA and JA-Ile burst as well as induction of defenses, in *A. thaliana* remains as yet unidentified.

The conversion of the free JA into an amino acid conjugate is necessary to generate the bioactive phytohormone. This is catalyzed in the cytosol by the enzyme JASMONATE RESISTANT 1 (JAR1) (Staswick and Tiryaki 2004). Then, JA-Ile enters the nucleus and binds to the jasmonate binding F-box protein COI1 (Xie et al. 1998; Yan et al. 2009), which is part of the SCF^{COI1} complex harboring an E3 ubiquitin-conjugating enzyme (Chini et al. 2007; Thines et al. 2007). This interaction is potentiated by inositol pentakisphosphate (IP_5) (Mosblech et al. 2011; Sheard et al. 2010). Almost all JA-responses are controlled by so-called JAZ (JASMONATE-ZIM-DOMAIN) proteins acting as repressors. Upon JA-Ile binding, the COI1-JA-Ile subunit of the SCF^{COI1} complex interacts with JAZ proteins thereby forming a co-receptor complex (Sheard et al. 2010); JAZ proteins are subsequently ubiquitinated and targeted for proteasome-mediated degradation (Chini et al. 2007; Thines et al. 2007). This omission of repression leads to activation of the transcription factor MYC2 and subsequently the expression of JA-responsive genes such as *VSP2* and others (Wasternack and Hause 2013) and, as a consequence thereof, the onset of defense reactions.

Many herbivory and other stress-induced, jasmonate-mediated plant defense responses have been studied in Arabidopsis and other plants by using jasmonate insensitive *coi1* or various JA-biosynthesis mutants, including *jar1* (for an overview see Wasternack and Hause 2013). Here, the *jar1* mutant is of particular interest because JAR1 is the last enzyme in the biosynthetic pathway and directly generates the bioactive jasmonate, JA-Ile. Only JA-Ile but not JA is able to rescue jasmonate insensitivity in *jar1* mutants (Staswick and Tiryaki 2004). This demonstrated that the generation and the presence of JA-Ile are necessary for jasmonate-mediated responses in plants. Consequently, *jar1* mutants have been widely used to study jasmonate signaling. Unfortunately, there are only a few plant species, where such mutants or transgenic, silenced plants are available for such studies: *Arabidopsis thaliana* (Staswick et al. 1992), *Oryza sativa* (rice) (Riemann and Takano 2008), *Nicotiana attenuata* (Kang et al. 2006; Wang et al. 2007), *Solanum lycopersicum* (tomato) (Suza et al. 2010). Thus, in cases where transgenic approaches are not feasible and the respective mutants are not known, specific inhibition of, for example, JA-Ile accumulation or sensing might be helpful and a valuable alternative to investigate jasmonate signaling.

Here, we report that the calcium inhibitor, neomycin, specifically affects *S. littoralis* OS-induced JA-Ile accumulation in Arabidopsis leaves. As a consequence thereof, downstream jasmonate-responses are affected as well.

Methods and Materials

Chemicals Neomycin sulfate was purchased from Enzo life science (Lörrach, Germany) and Sigma-Aldrich (Taufkirchen, Germany). All jasmonates employed in this work were synthesized starting from methyl-jasmonate (MeJA, Sigma-Aldrich), containing an equilibrium mixture of (3*R*,7*R*), (3*S*,7*S*), (3*R*,7*S*), and (3*S*,7*R*) isomers (ratio ca 45:45:5:5), respectively. Thus, all four diastereomers were present in the final product. JA was synthesized from commercially available MeJA by saponification. JA-Ile was prepared from JA and L-isoleucine (Ile) following the procedure described by (Koch et al. 1999) for linolenic acid-isoleucine conjugate. The product was purified by flash chromatography (elution with chloroform-ethyl acetate-acetic acid (30:15:1, v/v/v)) to provide the JA-Ile conjugate as a mixture of isomers (406 mg; 76.2 % overall yield). (*N*-((±)-Tuberonoyl)-L-isoleucine (12-OH-JA-Ile) for the determination of the response factor was synthesized as follows: ozonolysis of MeJA, Wittig reaction to regenerate the double bond as described for (±)-methyl-12-hydroxy-jasmonate (Nakamura et al. 2006), subsequent saponification of the methyl ester, conjugation to Ile as described for JA-Ile and final deprotection of the hydroxyl group. The product was purified by flash chromatography (elution with ethyl acetate-isopropanol-acetic acid (32:2:1, v/v/v)) to provide 183 mg (41.5 % overall yield) of the desired product as a mixture of isomers.

Plants and Insects Arabidopsis thaliana seeds (ecotype Columbia) were used for experiments. Seeds were sown in 10 cm round pots and stratified for 3–4 d at 4 °C. Afterwards, plants were moved to ventilated growth rooms with constant air flow and 57-71 % humidity at 21 °C. Plants were grown at a distance of 30 cm from fluorescent light banks with 6 bulbs of cool white and 2 bulbs of wide spectrum lights at a 10 h light/14 h dark photoperiod and a light intensity of 90–130 μmol m^{-2} s^{-1}. Plants were shifted to wide spectrum light source after 3 wk, and all the experiments were done on 5-wk-old plants. Larvae of *Spodoptera littoralis* were hatched from eggs and reared on an agar-based optimal diet (Bergomatz and Boppre 1986). Temperature was kept at 23–25 °C with 8 h light/16 h dark cycles. For collection of OS, 4th instar *S. littoralis* larvae reared on artificial diet were fed on Arabidopsis leaves for 24 h prior to collecting OS on ice. OS were collected into glass capillaries by gently squeezing the larva with a forceps behind the head, which caused immediate regurgitation. The OS was centrifuged at 13,000 rpm for 2 min, and the cleared supernatant was diluted 1:1 with water (Vadassery et al. 2012a).

Plant Treatments All induction experiments were performed at 5 wk post germination at a vegetative growth stage. For experiments with insect oral secretions, wounding was done

with a pattern wheel (6 vertical motions) on either side of the leaf (Vadassery et al. 2012a). A total of 20 μl of fresh diluted OS were spread across all the holes on a single leaf. In control plants, water was added. For experiments with Ca^{2+} inhibitors the plants were wounded and pre-incubated with inhibitor for 15 min prior to application of OS at the same site. In control plants, H_2O was pre-incubated for 15 min followed by treatment with OS. Samples were harvested and stored in liquid nitrogen. All experiments were repeated three times independently. Four-wk-old plants were used for JA-Ile spraying experiments. JA–Ile was prepared as stock of 50 mM JA-Ile in 100 % ethanol. 200 μM of JA-Ile (mixture of isomers) in water (corresponding to a final ethanol concentration of 0.4 %) was sprayed on either side of whole rosette, and 3 ml were sprayed per plant. In gene expression studies, treatment was done for 16 h; in the controls, 0.4 % ethanol in water was sprayed. Neomycin (100 μM) was pre-sprayed 6 h prior to JA-Ile treatment in the dark. Control plants were sprayed with neomycin/water followed by 0.4 % ethanol during inhibitor experiments.

Ca^{2+} Measurements To measure changes in the cytosolic Ca^{2+} concentrations that can be induced by OS and, furthermore, any effects of the inhibitor neomycin, transgenic *A. thaliana* expressing the Ca^{2+}-sensing protein apoaequorin were used (Knight et al. 1997). Plants were grown in 10 cm pots for 4–5 wk. For Ca^{2+} measurements, a leaf disc was taken and reconstituted in 5 μM coelenterazine (P.J.K. GmbH, http://www.pjk-gmbh.de/) in the dark overnight at 21 C. Bioluminescence counts in leaf discs were recorded at 5 s intervals for 10–20 min, recorded as relative light units per second (RLU/sec) with a microplate luminometer (Luminoscan Ascent, version 2.4, Thermo Fischer Scientific, Dreieich, Germany). After a 1 min background reading, *S. littoralis* OS was added manually to the well, and readings in RLU were taken for 15–20 min. For experiments with Ca^{2+} inhibitors, the leaf disc was pre-incubated with inhibitor, for 15–30 min prior to application of OS. Calibrations were performed by estimating the amount of aequorin remaining at the end of the experiment by discharging all remaining aequorin in 1 M $CaCl_2$ and 10 % ethanol, and the counts were recorded for 5 min. The luminescence counts obtained were calibrated using the equation by (Rentel and Knight 2004).

Quantification of JA, JA-Ile, and 12-OH-JA-Ile The protocol from Vadassery et al. (2012a) was used for phytohormone quantification. Plant material was weighed (250 mg), frozen with liquid nitrogen, and samples were stored at −80 ˚C until used. For phytohormone analysis, finely ground leaf material was extracted with 1.5 ml of methanol containing 60 ng of 9,10-D_2-9,10-dihydrojasmonic acid, 60 ng D_4-salicylic acid, 60 ng D_6-abscisic acid (Santa Cruz Biotechnology, Heidelberg, Germany), and 15 ng of jasmonic acid-[$^{13}C_6$] isoleucine conjugate as internal standards. Phytohormones were quantified relative to the signal of their corresponding internal standards. The peak of the endogenous bioactive form of JA-Ile, (+)-7-*iso*-jasmonoyl-L-isoleucine was used for JA-Ile quantification (Fonseca et al. 2009). The authentic 12-OH-JA-Ile (*N*-((±)-tuberonoyl)-L-isoleucine was used for the determination of the response factor. 12-OH-JA-Ile was quantified on the MRM *m/z* 338/130 (DP -50 V, CE -30 V) using the signal of the internal standard, jasmonic acid-[$^{13}C_6$] isoleucine conjugate, applying an experimentally determined response factor of 1.

JA conjugation Assay Arabidopsis *JAR1* (*GH11*) cDNA in pGEX-4 T-1 bacterial expression cassette was obtained from P. E. Staswick. The wild-type glutathione-S-transferase (GST)-JAR1 fusion protein was produced as described previously (Staswick and Tiryaki 2004; Staswick et al. 2002). GST bind Fractogel Catridges (Novagen, Darmstadt, Germany) were used for purification of JAR1-GST fusion proteins according to manufacturers protocol. The conjugation assay was performed with purified GST-JAR1 fusion protein.

Activity assays were performed to a total volume of 100 μl with 50 mM Tris–HCl (pH 8.3), 3 mM $MgCl_2$, 3 mM ATP solution, 1 mM DTT, 1 mM JA in 50 % methanol, and 1 mM L-amino acids. Ten μl of purified JAR1-GST protein elute (2 μg/μl) were added to the master mix along with amino acids and incubated for different time points 0.5, 1, 2, and 24 h at 25 °C. When neomycin (500 μM) was added, it was pre-incubated to mixture for 15 min prior to JA and amino acid addition. Water was used as a control treatment in other samples. The reaction was stopped by adding 5 μl of 1 M HCl. JA and JA conjugates were extracted with 100 μl ethyl acetate. After the assay, the sample was diluted 1:1000 fold with methanol spiked with phytohormone internal standards containing 100 ng/ml 9,10-D_2-9,10-dihydrojasmonic acid, and 20 ng/ml jasmonic acid-[$^{13}C_6$] isoleucine conjugate. The phytohormones were quantified as described earlier.

Expression Analysis by Real Time PCR Leaf material was ground to a fine powder in liquid N_2, and total RNA was isolated using the TRIzol Reagent (Invitrogen, Darmstadt, Germany) according to the manufacturers' protocol. An additional DNAse (Turbo DNAse, Ambion, Karlsruhe, Germany) treatment was included to eliminate any contaminating DNA. RNA quantity was determined photospectrometrically. DNA-free total RNA (1 μg) was converted into single-stranded cDNA using a mix of oligo-dT_{20} primers using the Omniscript cDNA synthesis kit (Qiagen, Hilden, Germany). Gene-specific primers (placed at the exon-exon junction for specific amplification of cDNA, whenever possible) were designed using the NCBI primer design tool (http://www.ncbi.nlm.nih.gov/tools/primer-blast). For real time PCR, primers producing 150 to 170 bp amplicons were used. Q-RT-PCR

was done in optical 96-well plates on a Real-Time PCR Detection System (Biorad, München, Germany) using the Brilliant QPCR SYBR green Mix (Agilent Technologies, Böblingen, Germany) to monitor double-stranded DNA synthesis. *RPS18B* performed best as an endogenous control ('normalizer') upon herbivory. Thus, the mRNA levels for each cDNA probe were normalized with respect to the *RPS18B* mRNA level. Fold induction values of target genes were calculated with the ΔΔCP equation (Pfaffl 2001) and related to the mRNA level of target genes in control leaf, which were defined as 1.0. All of the assays were run in triplicate (biological replication) to control for overall variability. The primer pairs used are listed below:

RPS18B (*At1g 34030*):
5'-GTCTCCAATGCCCTTGACAT -3'; 5'-TCTTTCCTCTGCGACCAGTT-3'
LOX2 (At3g45140):
5'-ACGCTCGTGCACGCCAAAGT-3'; 5'-TCCTCAGCCAACCCCCTTTTGA-3'
AOS (At5g42650):
5'-AAGCCACGACGCGGCGTTTA-3'; 5'-GGAGTCTCCGTCTCCGGTCCA-3'.
MYC 2 (At1g 32640):.
5'-CGGAGATCGAGTTCGCCGCC-3'; 5'-AATCCCGCACCGCAAGCGAA-3'.
JAZ10 (At5g 13220):.
5'-TCGAGAAGCGCAAGGAGAGATTAGT-3'; 5'-AGCAACGACGAAGAAGGCTTCAA-3'.
PDF1.2 (At5g44420):.
5'-CTGCTTTCGACGCACCGGCA-3'; 5'-GTTGCATGATCCATGTTTGGCTCCT-3'.
Thi2.1 (At1g72260):.
5'-CGCCATTCTCGAAAACTCAGCTGA-3'; 5'-GTTTAGGCGGCCCAGGTGGG-3'.
VSP2 (At5g24770):
5'-ACGACTCCAAAACCGTGTGCAA-3'; 5'-CGGGTCGGTCTTCTCTGTTCCGT-3'.
CYP94B3 (At3g48520):.
5'-ATGGACCACCATCGTATCCACTCA-3'; 5'-GCGACGGTTGCCGAGGAGAG-3'.

Amino Acid Measurements The methanolic extracts from phytohormone extraction (see above) were diluted in a ratio of 1:20 (v:v) in water containing the ^{13}C-, ^{15}N-labelled algal amino acid mix (Isotec, Miamisburg, OH, USA) at a concentration of 10 μg of the mix per ml. Amino acids in the diluted extracts were directly analyzed by LC-MS/MS. The analysis method was modified from a protocol described by (Jander et al. 2004). Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm, Agilent Technologies). Formic acid (0.05 %) in water and acetonitrile were employed as mobile phases A and B, respectively. The elution profile was: 0–1 min, 3 % B in A; 1–2.7 min, 3-100 % B in A; 2.7-3 min, 100 % B; and 3.1-6 min, 3 % B in A. The mobile phase flow rate was 1.1 ml/min. The column temperature was maintained at 25 °C. The liquid chromatograph was coupled to an API 5000 tandem mass spectrometer (Applied Biosystems, Darmstadt, Germany) equipped with a Turbospray ion source operated in positive ionization mode. The instrument parameters were optimized by infusion experiments with pure standards (amino acid standard mix, Fluka, St. Louis, MO USA). The ionspray voltage was maintained at 5500 eV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 70 psi, curtain gas at 35 psi, heating gas at 70 psi, and collision gas at 2 psi. Multiple-reaction monitoring (MRM) was used to monitor analyte parent ion→product ion: MRMs were chosen as in Jander et al. (2004). Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies were verified by analyzing dilution series of standard mixtures (amino acid standard mix, Fluka plus Gln, Asn and Trp, also Fluka). The concentration of the individual labelled amino acids in the mix had been determined by HPLC-fluorescence detection analysis after pre-column derivatization with ortho-phthaldialdehyde-mercaptoethanol using external standard curves made from standard mixtures. Individual amino acids in the sample were quantified by the respective ^{13}C, ^{15}N-labelled amino acid internal standard.

Statistical Analysis Statistical differences between different groups were detected by unpaired students *t*-test and one-way ANOVA and *post hoc* SNK test in SigmaStat 2.03. Different letters indicate significant difference between treatments.

Results

Neomycin Affects JA-Ile but not JA Accumulation In Arabidopsis leaves, within minutes the *Spodoptera littoralis*-derived OS application induces both a transient Ca^{2+} increase and a JA/JA-Ile burst (Vadassery et al. 2012a). The hypothesis was tested that blocking the Ca^{2+} response has an effect on *S. littoralis* OS-induced phytohormone elevation. Therefore, various well known Ca^{2+} antagonists (*e.g.*, BAPTA, La^{3+}, ruthenium red, neomycin) were tested for their action on both OS-induced Ca^{2+} response and phytohormone level changes. Among them, neomycin showed striking results. Neomycin blocks the release of Ca^{2+} from internal stores (Tang et al. 2007). Hence, 15–30 min long pre-treatment with 100 μM neomycin followed by application of *S. littoralis* OS was

performed on Arabidopsis leaves. Neomycin pre-treatment significantly reduced the *S. littoralis* OS-induced Ca^{2+} elevation (Fig. 1) although the initial Ca^{2+} peak was not completely blocked. Jasmonate changes were further measured upon different treatments: wounding+H_2O (W+W), wounding+neomycin (W+N), wounding+*S. littoralis* OS (W+OS), and wounding+neomycin+OS (W+N+OS). Neomycin was pre-incubated on the wounded leaf for 15 min prior to *S. littoralis* OS application. It was previously found that *S. littoralis* OS induced a phytohormone burst, and JA-Ile accumulated transiently reaching a peak at 30–60 min and declining gradually to resting levels (Vadassery et al. 2012a). Upon OS application, JA levels increased gradually reaching a peak at 60 min and then decreased gradually. Neomycin pretreatment, however, altered this trend. After 30 min, JA-Ile elevation was significantly reduced due to neomycin pre-treatment (Fig. 2a). Surprisingly, JA elevation was unaffected by neomycin pre-treatment (Fig. 2b) suggesting that neomycin specifically affects JA-Ile.

Reduced JA-Ile Accumulation Is not Due to Limited Substrate Availability or Defective Conjugation Process Reduced JA-Ile levels upon neomycin treatment followed by OS application could be due to two reasons- a) unavailability of substrates (Ile or JA) for JA-Ile biosynthesis or b) impaired JAR1 enzyme activity that affects the conjugation of JA to JA-Ile. Because neomycin specifically reduced only JA-Ile levels, whereas JA upon OS application was unchanged, JA level was not the limiting substrate. Thus, the amount of isoleucine (Ile) was quantified upon W+W, W+N, W+OS, and W+OS+N treatments. The Ile levels were similar upon W+W and W+N treatments, and increased again to a similar level after W+OS and W+OS+N treatments (Fig. 3a). This implies that the level of Ile was not limiting for JA-Ile formation. Next, the conjugation process of JA to JA-Ile by JAR1 was examined in the presence and absence of neomycin, using the JA-amino acid conjugation enzyme assay (Staswick and Tiryaki 2004) and by further quantification of JA-Ile formed by LC-MS/MS in a time course dependent manner at 0.5, 1, 2, and 24 h in the presence and absence of neomycin. However, addition of neomycin to the enzyme assay did not affect the conjugation process, and JA-Ile levels were unchanged in the assay (Fig. 3b).

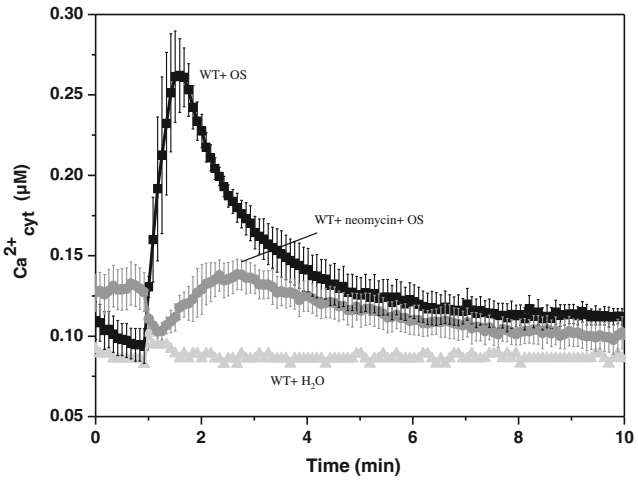

Fig. 1 *Spodoptera littoralis* oral secretions (OS)-induced changes in $[Ca^{2+}]_{cyt}$ concentrations. Application of 40 μl OS (1:1 diluted) to 5-wk-old Arabidopsis leaf discs expressing the Ca^{2+} reporter, aequorin, with and without pre-treatment (15 min) with 100 μM neomycin prior to OS application. Mean (±SE, N=4) $[Ca^{2+}]_{cyt}$ was calculated from the relative light units measured in leaf disc at 5 s integration time for 10 min from 3 independent experiments. In all the experiments, water was used as control and gave background readings

JA-Ile Turnover via CYP94B3 The ω-oxidation pathway catalyzed by two cytochrome P450 enzymes, CYP94B3 and CYP93C1, in which JA-Ile is converted to12-hydroxy-JA-Ile (12-OH-JA-Ile) and then further oxidized to 12-carboxy-JA-Ile (12-COOH-JA-Ile), is a major route for catabolism of the hormone (Heitz et al. 2012; Kitaoka et al. 2011; Koo et al. 2011). Therefore, the hypothesis was tested that JA-Ile levels would decrease upon neomycin treatment, because the turnover of JA-Ile is affected. Transcript analysis after 60 min of treatment revealed that *CYP94B3* expression was more up-regulated upon W+OS+N treatment (174-fold), compared to W+OS (86-fold) and W+W (54-fold) (Fig. 4b). However, the *CYP94B3* expression at 30 min treatment remained unchanged between treatments (Fig. 4a). Further, the levels of 12-OH-JA-Ile formation were measured over a time course of 30, 60, 120, and 180 min upon W+W, W+N, W+OS, and W+OS+N treatments to examine if changes in gene expression correlated with 12-OH-JA-Ile accumulation (Fig. 4c). In general, 12-OH-JA-Ile accumulation was delayed compared to the JA-Ile burst, and at 30 min all treatments gave similar levels. A significant difference between the W+W and W+OS treatments was visible only from 120 min on. Surprisingly, 12-OH-JA-Ile levels between W+OS and W+OS+N showed no difference at any of the time points tested (Fig. 4c). Next, the ratio of JA-Ile to 12-OH-JA-Ile level over the time points was calculated (Fig. 2a, 4c). At the 30 min time point, the ratio was 0.38 for W+W, 0.49 for W+N, and, most importantly, 0.81 for W+OS but only 0.47 for W+OS+N, which was similar to background levels. So upon neomycin treatment, the ratio of JA-Ile to 12-OH-JA-Ile formation was significantly reduced, though the absolute levels of 12-OH-JA-Ile did not change.

JA-Ile-induced Gene Expression Is Affected by Neomycin Following JA-Ile perception by its receptor, JA responsive genes responsible for plant defense are reported to be induced, but only a few experiments are based on JA-Ile treatment. As a consequence thereof, JA-Ile was chemically synthesized and sprayed to Arabidopsis to identify the

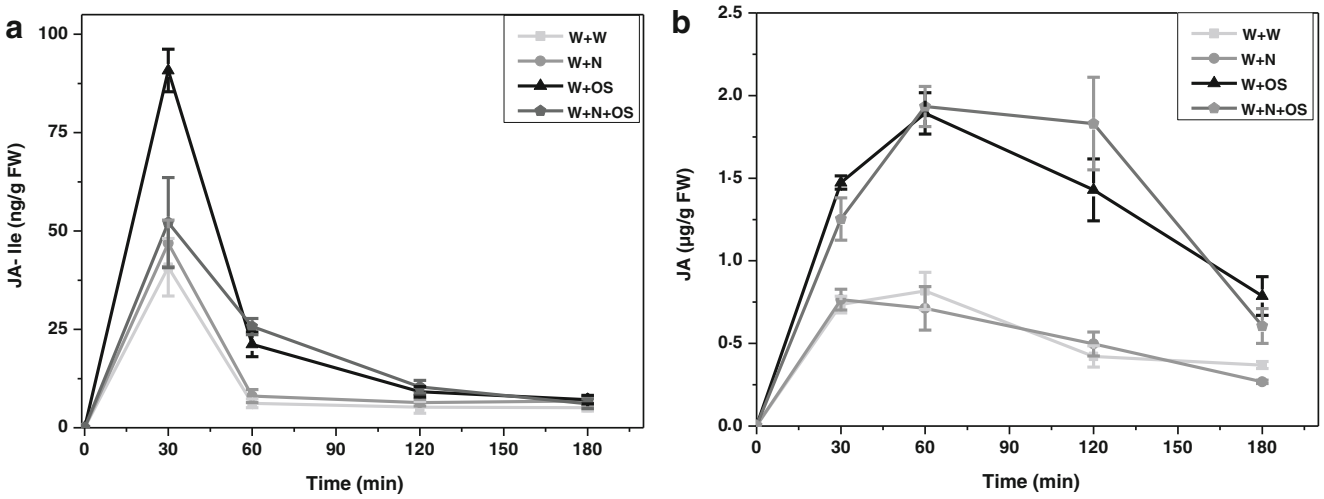

Fig. 2 Kinetics of *Spodoptera littoralis* OS-induced JA-Ile and JA levels in 5-wk-old *Arabidopsis thaliana* leaves upon neomycin treatment. Mean (±SE, *N*=3) content of JA-Ile (**a**) and JA levels (**b**) at time points 30, 60, 120, and 180 min after wounding and treatment with H_2O, *S. littoralis* OS, neomycin, and neomycin+OS. Leaves were wounded by pattern wheel and pre-treated once with either 100 µM neomycin or water for 15–30 min. Subsequently 20 µl water or 20 µl 1:1 diluted OS per leaf were added. Phytohormones were measured in local treated leaves

specific genes induced by JA-Ile. It was found that the JA-responsive gene, *VSP2,* was highly up-regulated (240-fold) 16 h after JA-Ile spraying (Fig. 5a). Other JA-responsive genes like *Thi2.1*, *PDF1.2,* transcription repressor *JAZ10,* JA biosynthetic genes like *LOX2*, *AOS* were up-regulated upon JA-Ile treatment at varying but much lower levels. Surprisingly, JA-Ile catabolizing *CYP94B3* did not show a transcriptional up-regulation after 16 h treatment with JA-Ile (Fig. 5b). The effect of neomycin on gene expression pattern of the JA-Ile responsive genes was investigated as well. Neomycin pre-treatment for 6 h followed by JA-Ile treatment (16 h) resulted in a striking inhibition of JA-Ile responsive gene (*VSP2)* (Fig. 5a) and JA biosynthetic gene (*LOX2)* (Fig. 5b). However, transcriptional repressor (*JAZ10)*, JA biosynthetic gene (*AOS),* and *CYP94B3* were all strongly up-regulated upon neomycin+JA-Ile treatment compared with JA-Ile alone (Fig. 5b). The *CYP94B3* up-regulation was similar to its action upon N+*S. littoralis* OS treatment shown in Fig. 3a. However, expression of *MYC2*,

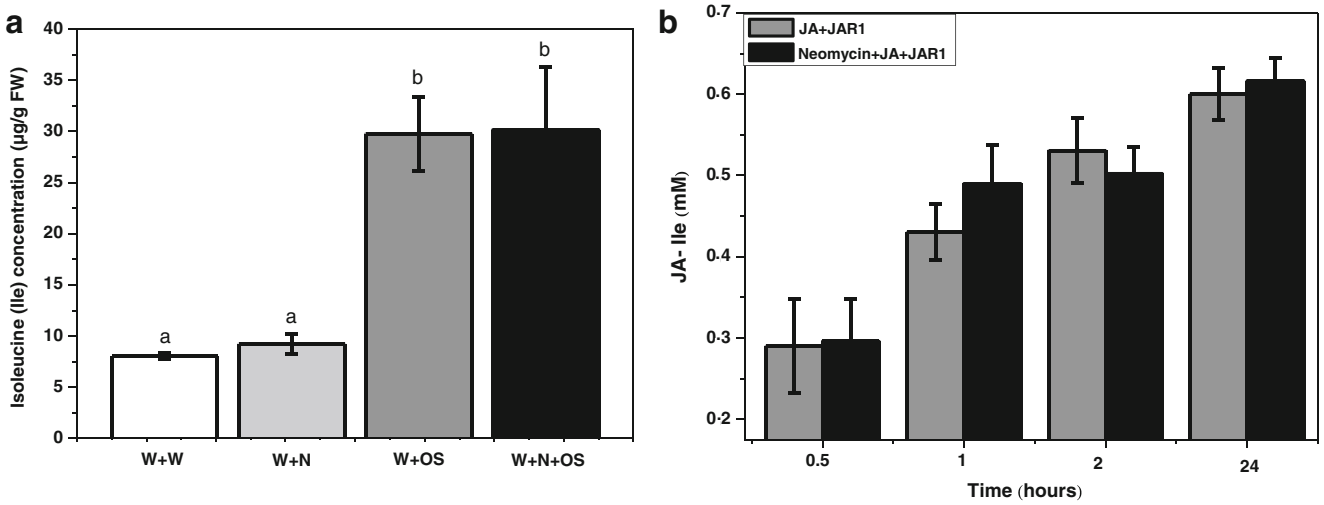

Fig. 3 Neomycin pre-treatment effects on isoleucine concentration and JA to JA-Ile conjugation. **a** Mean (±SE, *N*=7) content of amino acid, isoleucine at 60 min after wounding+H_2O (W+W), wounding+neomycin (W+N), wounding+*Spodoptera littoralis* OS (W+OS), and wounding+neomycin+OS (W+N+OS) treatments. Wounded leaves of 5-wk-old *Arabidopsis thaliana* plants were pre-treated with 100 µM neomycin or water for 15–30 min before OS application. Isoleucine was measured in local treated leaves. **b** Mean (±SE, *N*=8) concentration of JA-Ile formed after JAR1 conjugation assay, upon incubation of JA+JAR1 enzyme and neomycin+JA+JAR1 for 0.5, 1, 2 and 24 h time points

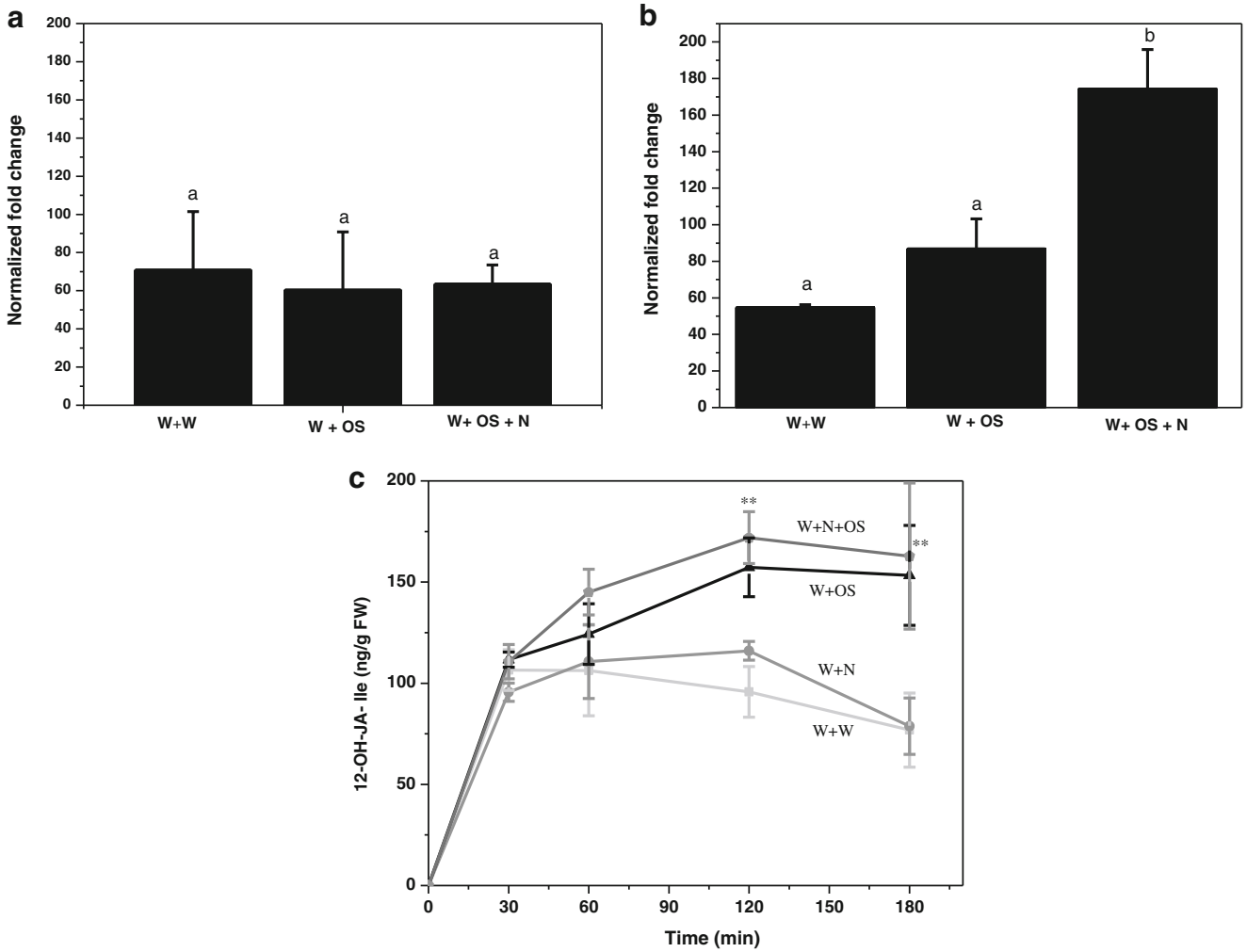

Fig. 4 **a**,**b** *CYP94B3* transcript levels in 5-wk-old *Arabidopsis thaliana* leaves upon wounding+H_2O (W+W), wounding+*Spodoptera littoralis* OS (W+OS) and wounding+neomycin+OS (W+N+OS) treatments for 30 (**a**) and 60 min (**b**). Leaves were wounded by pattern wheel and pre-treated once with either 100 μM neomycin or water for 15–30 min, before the OS application. Transcript abundance in leaves was determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. The graph shows mean (±SE, *N*=3) of x-fold change calculated relative to control which was unwounded leaf. Different letters indicate significant differences among treatments (ANOVA, SNK *post hoc* test: $P<0.05$) and experiment was repeated 2 times independently. **c** Mean (±SE, *N*=3) content of 12-OH-JA-Ile levels at different time points after wounding+H_2O (W+W), wounding+neomycin (W+N), wounding+*S. littoralis* OS (W+OS), and wounding+neomycin+OS (W+N+OS) treatments. Leaves were wounded by pattern wheel and pre-treated once with either 100 μM neomycin or water for 15–30 min. Subsequently 20 μl water or 20 μl 1:1 diluted OS per leaf were added. Phytohormones were measured in local treated leaves. ** indicate significance at $P<0.01$

PDF1.2 and *Thi2.1* remained unchanged upon neomycin pre-treatment (Fig. 5b).

Discussion

In Arabidopsis, application of *S. littoralis*-derived OS has been shown to induce early responses, among them cytosolic Ca^{2+} elevation and downstream jasmonate elevation (Vadassery et al. 2012a). The jasmonate pathway plays a dominant role in plant defense against insect herbivores. $[Ca^{2+}]_{cyt}$ elevation is due to an influx of Ca^{2+} from the extracellular medium, or by Ca^{2+} mobilization from intracellular compartments, and/or by both (Lecourieux et al. 2006). Neomycin, a poly-cationic aminoglycoside antibiotic, often is used as an internal Ca^{2+} store blocker. Described as a non-specific inhibitor of phospholipase C (PLC) it affects inositol triphosphate (IP_3) formation. IP_3 is one of the best characterized effectors of Ca^{2+} release from internal stores in animal cells, whereas in plant cells both IP_3 and IP_6 are effective (Lemtiri-Chlieh et al. 2003; Peiter 2011). Although inhibitor-studies generally should be handled with care, neomycin effects are well-described in different plant systems. For example, neomycin was able to completely inhibit IP_3 levels and Ca^{2+} transients in *Papaver rhoeas* and Arabidopsis (Franklin-Tong et al. 1996; Tang et al. 2007). In *Nicotiana* as well as soybean (*Glycine max*) cells, cytosolic Ca^{2+} responses

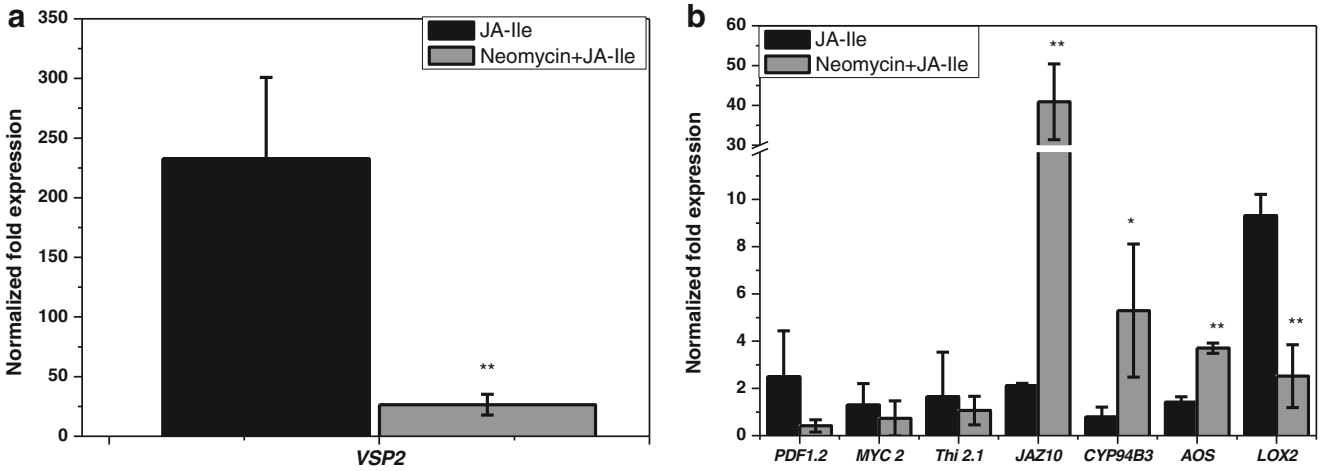

Fig. 5 Neomycin effect on JA-Ile induced gene expression. **a** Mean (±SE, *N*=4) of *VSP2* transcript levels with (grey) and without (black) neomycin (100 μM) pre-treatment before JA-Ile spray. Neomycin was pre-sprayed 6 h before treatment with JA-Ile (grey) for 16 h. Transcript abundance in 4-wk-old *Arabidopsis thaliana* leaves was determined by real-time PCR analysis and normalized to the plant *RPS18B* mRNA level. The fold change was calculated relative to control, which is a plant sprayed with 0.4 % ethanol, and neomycin+0.4 % ethanol. **b** Mean of (±SE, n=4) *PDF1.2, MYC2, Thi2.1, JAZ10, CYP94B3, AOS,* and *LOX2* transcript levels with (grey) and without (black) neomycin pre-treatment before JA-Ile spray as in Fig. 4. ** indicate significance at $P<0.01$ and * indicate significance at $P<0.05$ by *t*-test

induced by various elicitors were strongly affected by neomycin (Cessna et al. 2007; Mithöfer et al. 1999; Vatsa et al. 2011). Moreover, neomycin also is known to regulate the slow vacuole (SV) channel, a nonselective voltage-dependent cation channel in the vacuolar membrane of plant species (Scholz-Starke et al. 2006). The antibiotic properties of neomycin against bacteria rely mainly on its ability to interact with certain features of prokaryotic 16S rRNA, thereby affecting ribosomal function and protein synthesis (Moazed and Noller 1987; Woodcock et al. 1991). Unspecific adsorption to other cell components is discussed as well (Padilla and Burgos 2010).

Although is it conceivable that in our approach neomycin acts directly on OS-provided bacteria rather than on the plant, and all neomycin effects we observed in Arabidopsis are indirect, such a scenario seems unlikely. On the one hand, several examples are documented in the literature where neomycin was able to inhibit for example elicited Ca^{2+} responses in sterile cell cultures, thereby excluding the impact of bacteria in such reactions (Mithöfer et al. 1999; Vatsa et al. 2011; Cessna et al. 2007). On the other hand, the data presented in Fig. 5 clearly show neomycin effects without any treatment with OS as a potential source for microbes.

Here, we probed to determine if neomycin affects OS-induced Ca^{2+} levels as well as downstream processes like phytohormone elevation. Surprisingly, neomycin not only strongly impaired the *S. littoralis* OS-induced cytosolic Ca^{2+} increase (Fig. 1) but also inhibited JA-Ile accumulation. Strikingly, the accumulation of JA was unaffected. However, a single application of neomycin to wounded leaf has no long term effect on JA-Ile levels; it can only be seen at early time points (Fig. 2a). In order to study the reason for the specific action of neomycin on JA-Ile levels, the hypotheses were tested that the amino acid substrate for JA-Ile formation, *i.e.*, isoleucine concentration, or the conjugation process itself, which is mediated by JAR1, may be affected. Neither decreased isoleucine concentrations nor could any inhibition of the conjugation process *per se* be detected (Fig. 3a,b). The levels of JA-Ile also are controlled by catabolism and deactivation of the hormone. Synthesis of 12-OH-JA-Ile, catalyzed by the CYP94B3 ω-oxidation pathway, contributes to a partial switch-off in jasmonate signaling and is responsible for the rapid turnover of JA-Ile that is formed during herbivory or wounding (Heitz et al. 2012; Kitaoka et al. 2011; Koo et al. 2011). Neomycin pre-treatment followed by OS application interestingly increased the expression of *CYP94B3*, however, only after 1 h (Fig. 4b), and this might be a reason for the increased turnover of JA-Ile and, as a consequence, reduction in its levels. Surprisingly, the increased gene expression did not result in increased levels of 12-OH-JA-Ile upon OS+N- treatment (Fig. 4c). However, strikingly, the ratio of JA-Ile to 12-OH-JA-Ile, (JA-Ile levels/12-OH-JA-Ile levels- from Figs. 2a and 4c at 30 min, the time point when neomycin action is prominent) was 0.81 for W+OS and 0.47 for W+OS+N. Obviously, the latter is similar to background levels. Hence, one of the reasons for decreased JA-Ile levels upon OS treatment after neomycin treatment is partially due to its action on *CYP94B3* and increased JA-Ile turnover. There also is the additional possibility that further fast enzymatic conversion of 12-OH-JA-Ile

in the ω-oxidation pathway makes its quantification difficult. In addition, the amidohydrolases IAR3 and ILL6 may also contribute by the cleavage of JA-Ile as well as of 12-OH-JA-Ile, which was demonstrated recently for wound-treated Arabidopsis leaves (Widemann et al. 2013). However, gene expression of both IAR3 and ILL6 strongly depends on the COI1/jasmonate pathway (Widemann et al. 2013). This latter finding questions whether the amidohydrolases can be involved in decreasing JA-Ile conjugates because neomycin already inhibits JA-Ile accumulation (Fig. 2a) and downstream processes. For *Nicotiana attenuate,* a similar enzymatic activity of a jasmonoyl-L-isoleucine hydrolase 1 (JIH1) has been described that is able to hydrolyze JA-Ile and can contribute to JA-Ile homeostasis (Woldemariam et al. 2012). Based on these considerations, it is conceivable that hydrolyzing as well as hydroxylation/carboxylation reactions contribute to JA-Ile catabolism. In order to clarify the mechanism of how neomycin suppresses induced JA-Ile accumulation in Arabidopsis, all these possibilities need to be tested in the near future by employing various mutants such as *cyp94b3,* cyp94b3cyp94c1, *iar,* and *ill* (Heitz et al. 2012; Widemann et al. 2013).

Due to the fact that inositolphosphates (IPs) seem to play an important role in jasmonate perception, the usage of neomycin was extended to investigate JA-Ile-induced gene expression. In contrast to most studies that have not used JA-Ile for gene expression analysis, here, selected transcript profiles obtained upon specifically spraying JA-Ile onto Arabidopsis leaves were analyzed. *VSP2* was by far the most highly up-regulated gene, followed by *LOX2.* Other genes such as *PDF1.2, JAZ10*, and *AOS* were only slightly induced. Neomycin spraying on whole rosettes 6 h before JA-Ile treatment for 16 h resulted in down regulation of *VSP2* and *LOX2* (Fig. 5a,b). During insect herbivory, the JA pathway is rewired and the downstream ERF and MYC2 transcription factor pathways operate antagonistically, with activation of the MYC2 branch being beneficial for the plant, whereas activation of the ERF branch does not affect insect performance (Verhage et al. 2011). *VSP2* is a major marker gene for the COI1 dependent MYC2-regulated branch of the JA-Ile signaling pathway that is involved in herbivore defense (Verhage et al. 2011). *LOX2* is known to be controlled by Ca^{2+}, as shown by the gain-of-function mutant, *fou2,* which has increased LOX activity. The *fou2* mutant carries a missense mutation in the putative voltage sensor of the Two Pore Channel 1 gene (TPC1), which encodes a Ca^{2+}-permeant non-selective cation channel in the tonoplast (Bonaventure et al. 2007). *JAZ10, AOS,* and *CYP94B3* (the latter involved in catabolism of JA-Ile) were up-regulated upon JA-Ile+neomycin treatment compared to JA-Ile treatment alone (Fig. 5b). Thus, it is tempting to speculate that blocking internal store-released Ca^{2+} elevations partially affected downstream JA-Ile-induced gene expression by both down-regulating and up-regulating specific sub-sets of genes.

The action of neomycin on internal Ca^{2+} stores is thought to be indirect, as neomycin inhibits release of IP_3 *via* its action on phospholipase C (PLC) in Arabidopsis (Tang et al. 2007). However, a role for IPs is emerging as being important in jasmonate perception. IPs are shown to be involved in JA-mediated responses downstream of JA biosynthesis (Mosblech et al. 2008). IP_5 binding is crucial for the COI1-JAZ co-receptor complex to perceive JA-Ile with high sensitivity (Mosblech et al. 2011; Sheard et al. 2010;). All this points to the possibility that PLC and IPs as well as IPs-mediated Ca^{2+} release from internal stores might play a significant role during defense against herbivory. Unfortunately, in the current study, quantification of IP_3 and IP_6 was not possible for us due to its very low levels in rosette leaves, but further experiments on metabolism are necessary to address this hypothesis. From such experiments, we also will get insights in the mode of action of neomycin. Nevertheless, this inhibitor is a valuable tool to investigate role of Ca^{2+} in jasmonate signaling in plants.

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5. Article II

Synthesis, metabolism and systemic transport of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0

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Signal translocation

ABSTRACT

Jasmonates (JAs) are fatty acid derivatives that mediate many developmental processes and stress responses in plants. Synthetic jasmonate derivatives (commonly isotopically labeled), which mimic the action of the endogenous compounds are often employed as internal standards or probes to study metabolic processes. However, stable-isotope labeling of jasmonates does not allow the study of spatial and temporal distribution of these compounds in real time by positron emission tomography (PET). In this study, we explore whether a fluorinated jasmonate could mimic the action of the endogenous compound and therefore, be later employed as a tracer to study metabolic processes by PET. We describe the synthesis and the metabolism of (*Z*)-7-fluoro-8-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)octanoic acid (7F-OPC-8:0), a fluorinated analog of the JA precursor OPC-8:0. Like endogenous jasmonates, 7F-OPC-8:0 induces the transcription of marker jasmonate responsive genes (JRG) and the accumulation of jasmonates after its application to *Arabidopsis thaliana* plants. By using UHPLC–MS/MS, we could show that 7F-OPC-8:0 is metabolized in vivo similarly to the endogenous OPC-8:0. Furthermore, the fluorinated analog was successfully employed as a probe to show its translocation to undamaged systemic leaves when it was applied to wounded leaves. This result suggests that OPC-8:0 – and maybe other oxylipins – may contribute to the mobile signal which triggers systemic defense responses in plants. We highlight the potential of fluorinated oxylipins to study the mode of action of lipid-derived molecules *in planta*, either by conventional analytical methods or fluorine-based detection techniques.

1. Introduction

Oxylipins are a diverse group of lipid-derived signaling compounds that are present throughout the plant kingdom [1]. They are generated following oxidation of polyunsaturated fatty acids (FA) such as linoleic acid, linolenic acid, and hexadecatrienoic acid [2,3]. Jasmonates (JAs) are among the best characterized FA derivatives [4]. These metabolites mediate many developmental processes and stress responses in plants, including leave senescence, mechano-sensitive signal transduction, secondary metabolism and plant responses to wounding or herbivory [4–7]. Jasmonic acid (JA) is probably the most studied member of the JAs' family [4].

The JA biosynthetic pathway is well understood and many of the involved enzymes are well characterized [8,9]. It starts in the plastid with the release of linolenic and hexadecatrienoic acids from the plastidic glycerolipids. A 13-lipoxygenase (LOX) is capable of oxidizing linolenic acid to 13-hydroperoxy linolenic acid (13-HPOT), which can be metabolized to different classes of oxylipins (Fig. 1) [10]. The conversion of 13-HPOT to 12,13-epoxyoctadecatrienoic acid (12,13-EOT) by an allene oxide synthase (AOS) is the main transformation of 13-HPOT. The allene oxide cyclase (AOC) acts on 12,13-EOT to produce *cis*-(+)-12-oxo-phytodienoic acid (*cis*-OPDA) stereospecifically, which is the first jasmonate having the cyclopentanone ring and remarkable bioactivity. Further conversion of *cis*-OPDA implies its translocation from the chloroplasts to the peroxisomes. There, *cis*-OPDA reductase 3 (OPR3) reduces *cis*-OPDA to

Abbreviations: JA, jasmonic acid; *cis*-OPDA, *cis*-(+)-12-oxo-phytodienoic acid; JA-Ile, JA-L-isoleucine conjugate; 7F-OPC-8:0, (*Z*)-7-fluoro-8-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)octanoic acid; OPC-8:0, 8-((1*S*,2*S*)-3-oxo-2-((*Z*)-pent-2-en-1-yl)cyclopentyl)octanoic acid; JRG, jasmonate responsive genes; FA, fatty acids; LOX, 13-lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR3, *cis*-OPDA reductase 3; OPC-6:0, (*Z*)-6-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)hexanoic acid; OPC-4:0, (*Z*)-4-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)butanoic acid; ACX, acyl-CoA oxidase; MFP, multifunctional protein; KAT, L-3-ketoacyl-CoA thiolase; PET, positron emission tomography; MeJA, methyl jasmonate; 5F-OPC-6:0, (*Z*)-5-fluoro-6-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)hexanoic acid; 3F-OPC-4:0, (*Z*)-3-fluoro-4-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)butanoic acid; DAST, diethylaminosulfur trifluoride; PCC, pyridinium chlorochromate; PPTS, pyridinium *p*-toluenesulfonate; VSP2, vegetative storage protein 2; JAZ, jasmonate-ZIM-domain protein; GST1, glutathione-S-transferase 1; SCF^{COI1}, ubiquitin-ligase receptor complex; MRM, multiple reaction monitoring; HRMS, high resolution mass spectrometry.

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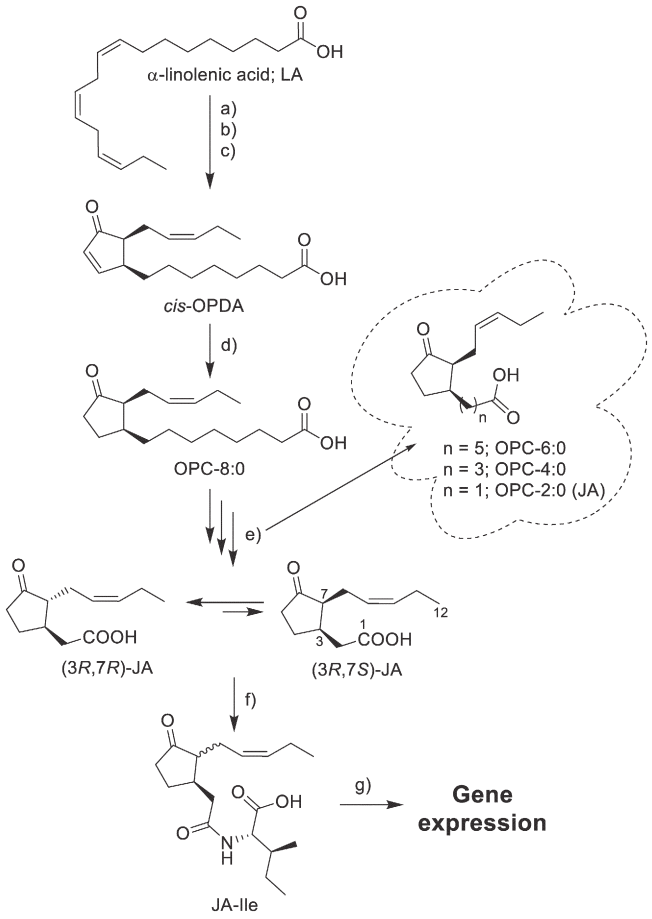

Fig. 1. Simplified scheme of the biosynthesis and signaling of jasmonates. In the plastids, LA is converted into *cis*-OPDA by the sequential action of LOX (a), AOS (b), and AOC (c). (3*R*,7*S*)-JA is formed in the peroxisomes by OPR3 (d) acting on *cis*-OPDA followed by three cycles of β-oxidation (e). (3*R*,7*S*)-JA can epimerize to the more stable isomer (3*R*,7*R*)-JA. In the cytosol, JA is conjugated to L-isoleucine (Ile) by jasmonic acid-amido synthetase (JAR1) to form the bioactive jasmonate JA-Ile (f), which can be subsequently perceived by the SCF^{COI1} co-receptor complex in the nucleus (g). This last process leads the expression of JRG and jasmonates induced responses. See text for detailed explanation and abbreviations. The compounds are shown in the stereochemistry occurring *in planta*.

8-((1*S*,2*S*)-3-oxo-2-((*Z*)-pent-2-en-1-yl)cyclopentyl)octanoic acid (OPC-8:0), lacking the highly reactive α,β-unsaturated keto group. Three rounds of β-oxidations are required for shortening the carboxyl side chain of OPC-8:0 producing OPC-6:0, OPC-4:0 and finally JA in that order (Fig. 1). The final product of the β-oxidations is (3*R*,7*S*)-JA (OPC-2:0), that can epimerize to the more stable isomer (3*R*,7*R*)-JA [4,11]. Both isomers co-exist *in planta* and we refer to them simply as JA.

Synthetic derivatives of jasmonates have been very helpful to elucidate the structural requirements for bioactivity, the biosynthetic and metabolic pathway of jasmonates [12–17]. These compounds, in particular isotopically labeled ones, have proven their utility to study transport phenomena in diverse plant species by different techniques (e.g., LC–MS and PET) [18]. For instance, after feeding wounded leaves with deuterium-labeled JA, [^{2}H]JA was translocated to systemic leaves and metabolized there to its ω-hydroxylated form 12-OH-JA [19]. It was later demonstrated that JA-Ile had a higher mobility than JA despite its lower polarity, and application of [^{2}H]JA-Ile to wounded leaves leads to a higher accumulation of JA and JA-Ile in distal leaves compared with control plants [20]. The translocation of methyl jasmonate (MeJA, **1**) was investigated by PET employing [^{11}C]MeJA as a tracer [21]. In this study it was claimed that **1** moves in both the phloem and xylem. However, it was shown later that the ester group (carrying the [^{11}C]) of MeJA (**1**) can be cleaved in vivo [22]. Therefore, further studies are required revisiting this topic.

Besides isotopically labeled compounds, fluorinated analogs have been widely employed to study biological processes. For example fluorinated FAs, provided very useful information on the structure-activity relationship, biosynthetic pathways, biological activities and metabolism of the target molecules [23,24]. Fluorinated derivatives of abscisic acid helped to gain insights into the biological activity and mechanism of activation and shutdown of this phytohormone [25]. Moreover, fluorine is a monoisotopic element (100% natural isotopic abundance) with a high gyromagnetic ratio ($\gamma = 40.05$ MHz/T). These properties make fluorinated molecules very interesting probes to be use in techniques like in HRMS and NMR. Interestingly, the fluorine chemistry of jasmonates remains little explored, although a few studies have dealt with fluorinated jasmonates. These compounds have shown different biological properties such as tuber-inducing effect in potato [26], anti-tumor action [27], and selective induction of plant secondary metabolites [28]. However, to the best of our knowledge, there are no physiological studies of fluorinated jasmonates described in the literature.

Herein we describe the synthesis of 7F-OPC-8:0 (**10**), a fluorinated derivative of the JA precursor OPC-8:0. We study its ability to induce JRG expression and jasmonates accumulation in *Arabidopsis thaliana* plants. Additionally, the metabolic fate of **10** in the plant and the possibility of using this molecule as a probe to follow signal-trafficking *in planta* is explored. Furthermore, a UHPLC–MS/MS method was developed to identify 7F-OPC-8:0 (**10**) and its metabolic derivatives in plant leaf extracts. This method was further employed to study whether **10** could be systemically translocated in the plant.

2. Results and discussion

2.1. Synthesis and characterization of 7F-OPC-8:0 (*10*)

7F-OPC-8:0 (**10**) was prepared as a mixture of isomers starting from commercially available MeJA (**1**). The synthesis was carried out according to the procedure depicted in Fig. 2. The fluorine atom was introduced at position C7 due to three main reasons (i) the replacement of a hydrogen atom by fluorine (similar atomic radius) should not cause steric hindrance or stereochemical restrictions in metabolic processes, (ii) to assures the tracking 7F-OPC-8:0 (**10**), 5F-OPC-6:0 (**11**) or 3F-OPC-4:0 (**12**), and no other JA derivatives when using fluorine-based imagine techniques such as PET and MRI, and (iii) the easy chemistry required for the introduction of the fluorine in that particular position.

The synthesis proceeded smoothly with moderate to high yields. Protection of the carbonyl group of the cyclopentane ring of **1**, followed by reduction of the ester group of **2** with $LiAlH_4$ in Et_2O, and oxidation of the alcohol **3** with pyridinium chlorochromate (PCC) afforded the aldehyde **4** in excellent yield (Fig. 2, steps a–c). Elongation of the side chain of **4** was carried out by both Grignard reaction and via the organolithium reagent derived from **6**. The second strategy was more efficient and provided cleaner products. Treatment of the alcohol **7** with diethylaminosulfur trifluoride (DAST) successfully afforded the fluorinated derivative **8**, which was deprotected without previous purification. Deprotection of both, the carbonyl and hydroxyl group of **8** was achieved in one single step by stirring **8** in a solution of Me_2CO:EtOH:water (1:1:1) containing pyridinium *p*-toluenesulfonate (PPTS). The fluorine containing alcohol **9** was obtained in 38% over two steps. Finally, treatment of **9** with Jones reagent harbored the fluorinated analog 7F-OPC-8:0 (**10**) (78%, mixture of isomers). As summary, **10** was obtained from MeJA (**1**) in six major transformations and 8% overall yield.

2.2. 7F-OPC-8:0 (*10*) induces the expression of JA-responsive genes (*VSP2, OPR3, JAZ1*) and cis-OPDA-responsive genes (*GST1, OPR1*)

The capability of 7F-OPC-8:0 (**10**) to induce both the accumulation of jasmonates and gene expression of marker JRG was evaluated upon

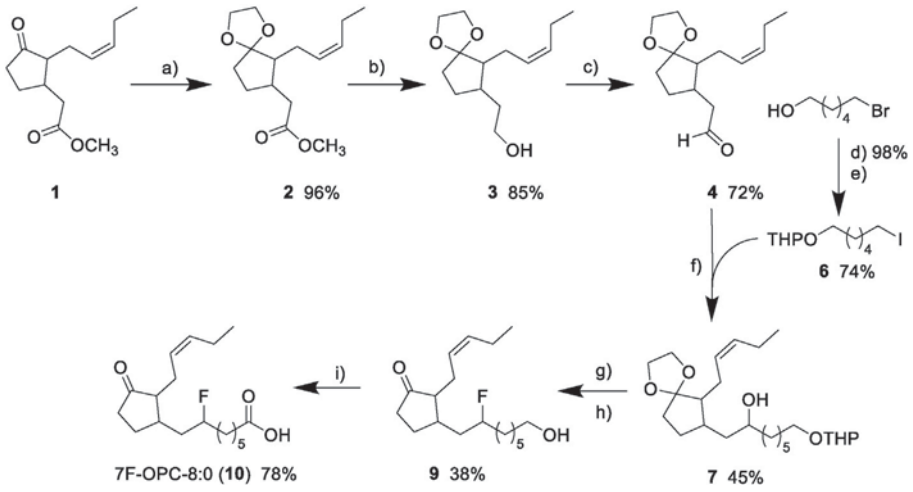

Fig. 2. Synthesis of 7F-OPC-8:0 (**10**). Reagents and conditions: a) 1,2-ethanediol/C_6H_6/*p*-TsOH, reflux; b) $LiAlH_4/Et_2O$; c) PCC/CH_2Cl_2/AcONa, 4 Å molecular sieves; d) NaI/Me_2CO; e) CH_2Cl_2/THP/*p*-TsOH, room temp.; f) *n*-pentane/Et_2O (3:2)/*t*-BuLi, −78 °C; g) DAST/CH_2Cl_2, −78 °C; h) Me_2CO/EtOH/H_2O (1:1:1)/PPTS; i) Jones reagent (4 M). For abbreviations see the text below.

application of the compound to *A. thaliana* plants. Jasmonates coordinate the plant responses to biotic and abiotic challenges by the induction of JRG expression, which is mediated by the SCF^{COI1}-JAZ co-receptor complex [29]. The activation of JRG is a typical plant response to herbivory [30–32] and mechanical wounding [33]. Activation of such genes also occurs after treatment with several endogenous jasmonates or synthetic analogs [31,34,35]. To evaluate 7F-OPC-8:0 (**10**) capability of inducing the expression of marker JRG, *A. thaliana* plants were sprayed with this compound and the transcript level of the selected genes was monitored. For this purpose two genes, strongly induced by JA were chosen: *VSP2* and *OPR3* [36]. Both genes are highly induced by 7F-OPC-8:0 (**10**) compared to solvent control, with a maximum peak at 30 min after treatment (Fig. 3 A,B). This result was consistent with similar analyses carried out with endogenous jasmonates [37].

On the other hand, some genes show a specific induction by the JA precursor *cis*-OPDA and are classified as *cis*-OPDA-responsive genes [36]. Two of which – *OPR1* and *GST1* – have been used as markers for *cis*-OPDA-responsive gene expression after wounding [38]. These genes are also highly upregulated after plant treatment with 7F-OPC-8:0 (**10**) (Fig. 3 C,D). Moreover, gene expression profiles observed upon 7F-OPC-8:0 (**10**) treatment are similar to the profiles observed for *cis*-OPDA (Fig. 1, reference [39]), which is directly metabolized in vivo to OPC-8:0 and finally JA, and thus, serves as a positive control for non-fluorinated jasmonates. Additionally, the gene coding for the transcriptional repressor of JA-signaling, JAZ1 [40], was induced after treatment with 7F-OPC-8:0 (**10**) (Fig. 2, reference [39]). All together those results suggest that 7F-OPC-8:0 (**10**) induces not only JA-responsive genes, but also genes responding to *cis*-OPDA, independent on the presence of the fluorine atom.

*2.3. 7F-OPC-8:0 (**10**) treatment-dependent increase of endogenous jasmonates levels, including cis-OPDA levels*

The activation of JRG is usually preceded by a transient increase in the internal levels of endogenous jasmonates [5]. As mentioned, compound **10** is capable of activating a subset of JRG (see Section 2.2). Accordingly, we expected that after plant treatment with 7F-OPC-8:0 (**10**), the jasmonates profile would be similar to the profile observed after simulated herbivory, wounding or jasmonates treatment. Fig. 4 shows the jasmonates profile for a time course experiment for *A. thaliana* plants treated with **10**. The concentrations measured for JA, JA-Ile, 11/12-OH-JA[2] and *cis*-OPDA showed the same trend (e.g. the JA/JA-Ile burst) observed in plants after simulated herbivory, JA treatment, or mechanical wounding. In order to examine the ability of **10** to initiate the accumulation of bioactive JA-Ile in comparison with *cis*-OPDA, both compounds were applied to *A. thaliana* leaves. Similar to the gene expression assays, the responses found for **10** again resembled the results found for *cis*-OPDA (Fig. 3, reference [39]).

This finding suggests that JAs downstream of OPC-8:0 in the metabolic pathway (Fig. 1) increase their level due to the in vivo metabolized 7F-OPC-8:0 (**10**). This suggestion agrees with that one postulated by Miersch and Wasternack for tomato plants [34]. These authors treated tomato leaves with deuterium-labeled OPC-8:0 and found that increasing JA and MeJA (**1**) levels were merely due to the metabolism of the deuterated applied compound, which is in line with our findings. In the same study, it was shown that the biosynthesis of jasmonates is not induced by treatment with jasmonates. Notwithstanding, we also found somewhat higher concentrations of *cis*-OPDA (1.2 fold) in plants treated with **10** compared to control plants after 1 h (Fig. 4D), although these differences were significant. A possible explanation to this observation is that, *in A. thaliana*, *cis*-OPDA can be produced from storage sources such as arabidopsides. These molecules contain *cis*-OPDA linked through an ester bond to a glycerol moiety [41]. Accumulation of arabidopsides has been reported during hypersensitive response and after wounding [42] in *A. thaliana*. The cleavage of the ester bonds of arabidopsides leads to an increase in free *cis*-OPDA, indicating a function for arabidopsides as storage of signal compounds that can prolong the JA-signaling [43]. To explore the possibility that arabidopsides could be the source for the increase in *cis*-OPDA level in our experiments, we analyzed the content of arabidopside A and B in the leaf extracts. A pronounced depletion of the content of arabidopsides A and B was observed at the same time frame in which the increase of *cis*-OPDA level occurred (Fig. 4, reference [39]). This supports that arabidopsides may represent the source for the increase in the *cis*-OPDA. Whether a similar phenomenon is observed with endogenous jasmonates needs further investigation. Until here, our data suggest that 7F-OPC-8:0 (**10**) can be metabolized by the plant like a true mimic of the JA precursor OPC-8:0.

[2] The analytical method does not distinguish between 11-OH-JA and 12-OH-JA.

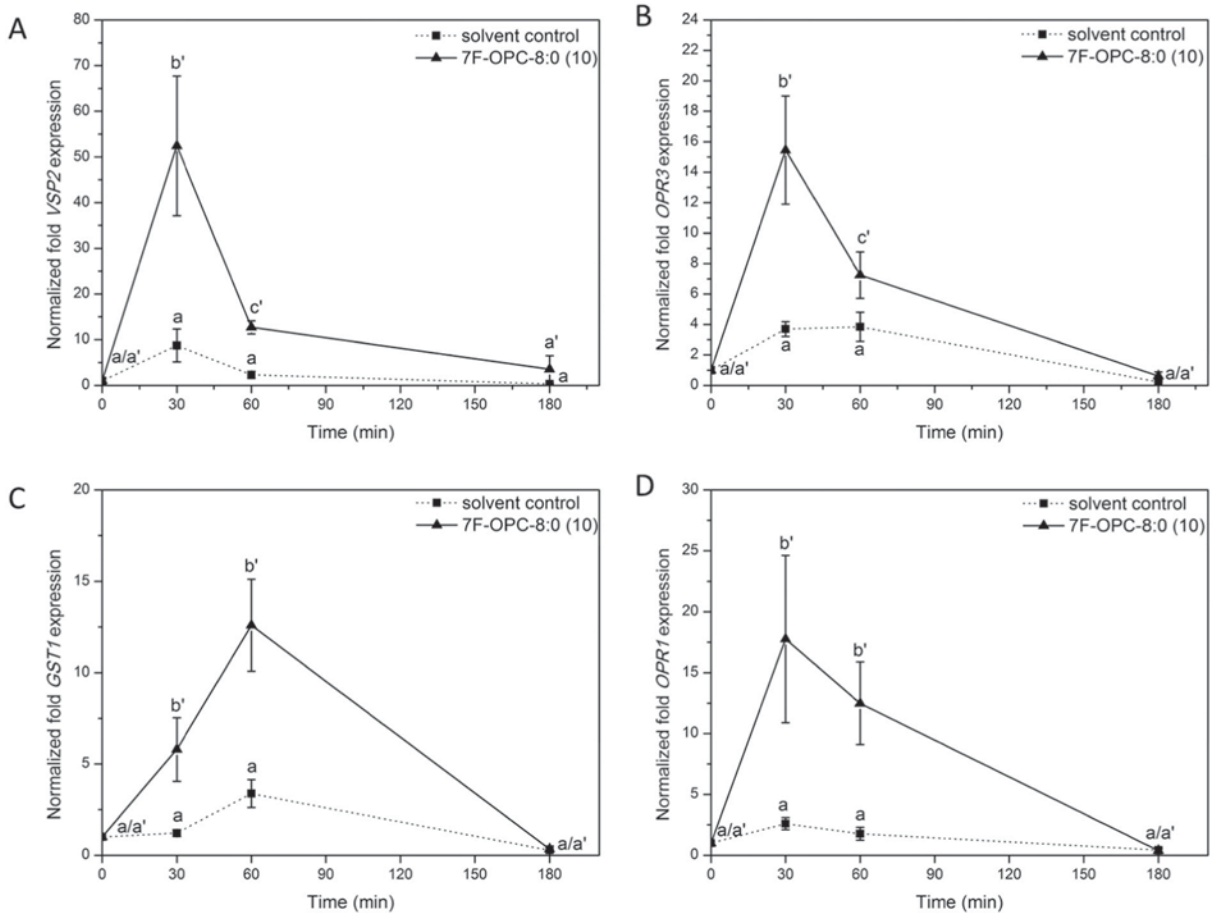

Fig. 3. Mean expression (±s.e., n = 5) of JA- (A, B) and *cis*-OPDA-responsive genes (C, D) in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. Expression of *VSP2* (A), *OPR3* (B), *GST1* (C) and *OPR1* (D) was analyzed after 30, 60 and 180 min. All samples were normalized to *RPS18B* level and untreated plants were used as control (expression level = 1). Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

*2.4. 7F-OPC-8:0 (**10**) is metabolized in vivo similar to the endogenous OPC-8:0*

Next, we addressed the question whether 7F-OPC-8:0 (**10**) can be metabolized by the plant like a true mimic of OPC-8:0, and may represent the source for the increased levels of jasmonates downstream to OPC-8:0 in the JA biosynthetic pathway (Fig. 4). In other words, we investigated whether **10** could undergo β-oxidations to produce JA.

An LC–MS/MS method was developed to identify **10** and the products resulting from its first two β-oxidations namely 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**). After the third β-oxidation the fluorine atom is lost. First, synthetic **10** was employed to fine tune the method in negative ionization mode on a Triple-Quadrupole mass spectrometer. The fragmentation pattern of **10** revealed that the molecular ion $[M–H]^-$, together with an intense peak resulting from a HF loss ($[M–H-20]^-$) are the most reliable fragments (Fig. 5, reference [39]). We were able to identify two signals corresponding to **11** and **12** in the samples of treated plants, by setting the quadrupole 1 (Q1) to $[M–H]^-$ and the quadrupole 3 (Q3) to $[M–H-20]^-$ in MRM mode. The identity of both peaks was corroborated by means of HRMS (Fig. 6, reference [39]). The concentrations found for **11** and **12** showed a similar profile to that observed for other jasmonates in this study (Fig. 5).

Our results show that 7F-OPC-8:0 (**10**) undergoes at least the first two β-oxidation steps similar to the endogenous OPC-8:0 in the JA biosynthetic pathway (Fig. 5). The presence of the fluorine atom does not hamper the oxidative degradation, as shown by the two fluorinated metabolites **11** and **12.** A third round of β-oxidation seems reasonable, since the amount of **12** slowly decreases after reaching a maximum. A detailed analysis of this step would require an additional label to follow the molecule after removal of fluorine.

2.5. 7F-OPC-8:0 (10) is systemically translocated in the plant

In response to wounding, plants accumulate jasmonates not only in wounded leaves but also in undamaged systemic leaves [5,20]. Currently it is not clear if this accumulation results from the direct transport, the *de novo* synthesis of the phytohormones or a combination of both events initiated by upstream signals [6]. Likewise, it is not well understood whether jasmonates including early precursors like OPC-8:0, may function as systemic signals in the plant.

We employed 7F-OPC-8:0 (**10**) as a probe to explore the possibility of this molecule being translocated and therefore involved in systemic signaling events in *A. thaliana* plants. The vascular connections between leaves are defined in *A. thaliana* plants [44]. Following the nomenclature of Farmer et al. [45], plants were mechanically wounded (pattern wheel) at leaf 8 of the *A. thaliana* rosette and **10** was immediately applied to the wounds. The contents of **10** and its derived metabolites 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) were determined in both damaged local and undamaged systemic leaves (Fig. 6).

In correspondence with previous results (Fig. 5, Section 2.4), **10** and its degradation products **11** and **12** could be measured in the treated leaf 8 (Fig. 6A). Interestingly, the levels of **11** and **12**were higher in the wounded leaf 8 than after application to an undamaged leaf. This could be explained by two reasons. On the one hand, the wounding

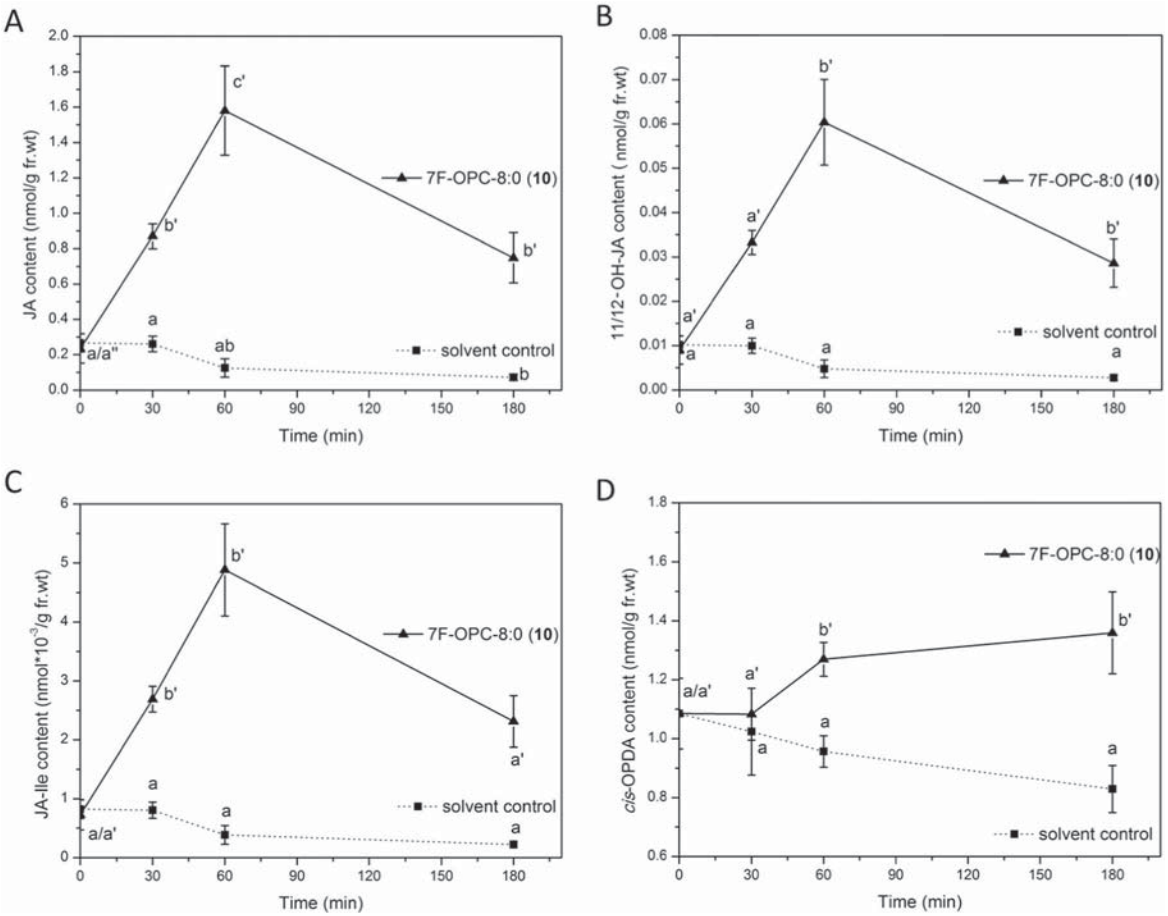

Fig. 4. Mean content (±s.e., n = 5) of jasmonate profiles in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. The content of JA (A), 11/12-OH-JA (B), JA-Ile (C) and *cis*-OPDA (D) was determined after 30, 60 and 180 min. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

effect can trigger the JA-biosynthesis in the damaged leaves [5], and consequently activate the jasmonate's metabolic machinery contributing to the metabolism of **10**. On the other hand, compound **10** could be assimilated easier through the wounds than when it is sprayed to unwounded tissue. 7F-OPC-8:0 (**10**) was detected both in younger (leaf 11) and older leaves (leaf 5), which are connected to leaf 8 via contact parastichies [44]. Leaves 5 and 11 were reported to react systemically when leaf 8 was wounded or feed by an insect, even though they do not share a direct vascular connection with leaf 8 [46,47]. The content of **10** in leaf 5 was significantly higher than in leaf 11. This is not surprising as some differences have been reported in the systemic response of these leaves [46]. The concentrations of **10** found in leaves 5 and 11 are in the same order of magnitude of those reported for JA-Ile systemically transported to distal leaves after wounding [5]. These results indicate that not only jasmonates but also their precursor OPC-8:0 is transported throughout the plant after wounding.

Interestingly, metabolites **11** and **12** were not detected in systemic leaves. A poor detection limit of the method employed could explain this; the levels of **11** and **12** in systemic leaves at the measured time point may be too low for detection. Therefore, neither a translocation of these compounds produced in leaf 8, nor a local synthesis from the translocated **10** can be ruled out. Further investigations are needed to clarify these questions. Based on these results, we conclude that the accumulation of jasmonates in systemic leaves is not only due to *de novo* synthesis of the phytohormones, but also an effect of the transport of JAs and precursors to the systemic undamaged tissue. Our data indicate that transport of OPC-8:0 occurs into older and younger leaves suggesting the action of these molecules as a systemic signal in a bidirectional way [44].

3. Conclusions

We developed a short synthesis of 7F-OPC-8:0 (**10**) – a fluorinated analog of the JA precursor OPC-8:0 – with good overall yield. This compound was shown to be active concerning the induction of marker JRG and accumulation of endogenous jasmonates in *A. thaliana* leaves. Furthermore, we were able to detect metabolites **11** and **12** derived from the β-oxidations of **10** in leaves extracts. As it has been demonstrated that application of jasmonates do not induce JA-biosynthesis, this suggests that externally applied jasmonates and analogs are metabolized to downstream JAs activating gene expression. Moreover, it has been demonstrated that the fluorinated analog **10** can be employed as a true mimic of the endogenous jasmonate OPC-8:0 in *A. thaliana* plants. We successfully employed 7F-OPC-8:0 (**10**) to show its translocation from damaged leaves to undamaged systemic leaves. This suggests that the JA precursors can also contribute to propagate systemic signals which induce defense responses of the plant in distal tissues to damaged area. Our results reveal the potential of the fluorine chemistry to study jasmonates – and optionally other phytohormones or plant lipid derivatives – metabolism and signaling. Plants are the energy source of many herbivorous organisms, therefore fluorinated jasmonates may be employed to study the metabolic fate of the fluorinated molecule in feeding organisms or even in tri-trophic interactions. Availability of compound **10** will allow the replacement of the fluorine atom by its

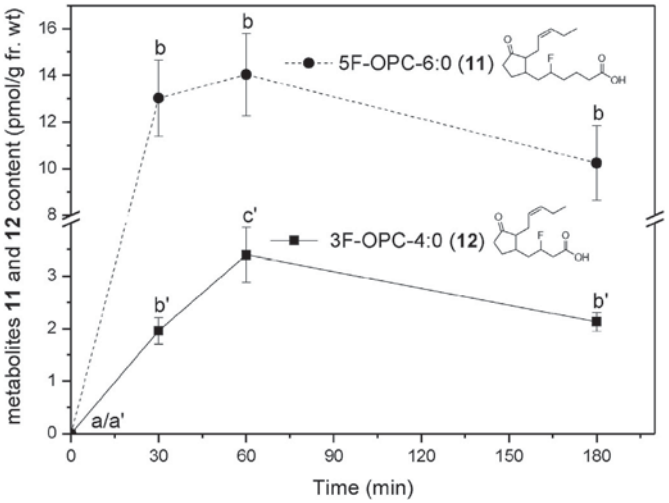

Fig. 5. Mean content (±s.e., n = 5) of the 7F-OPC-8:0 (**10**) derived metabolites 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) in *A. thaliana* Col-0 leaves after treatment with **10**. The content of 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) was determined after 30, 60 and 180 min. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

radioactive isotope ^{18}F to study transport phenomena in real time employing PET.

4. Material and methods

4.1. General material and methods

All chemicals were obtained from commercial suppliers. If necessary, solvents were purified prior to use. Thin layer chromatography was performed on silica gel 60 F_{254} on aluminum plates (Merck) and visualized with potassium permanganate staining. Flash chromatography was performed on silica gel 60 (40–63 μm) from Merck. Proportions of the employed solvents are referred to volume (*v*/*v*) if not mentioned otherwise.

GC-MS spectra were recorded on a ThermoQuest CE Instruments GC 2000 Series coupled to a ThermoQuest Finnigan Trace MS mass spectrometer; GC column HP-5MS capillary column (15 m × 0.25 mm ID with 0.25 μm film thickness, Phenomenex). Injection port: 250 °C; Split flow: 15 ml min^{-1} with split ratio of 1:10; Temperature program: 60 °C (2 min) at 15 °C min^{-1} to 280 °C (5 min). Helium at 1.5 ml min^{-1} served as carrier gas. The ionization method was electron impact (70 eV) in positive mode (EI^+). GC–MS for control of the chemical reactions was carried out on Hewlett Packard Series II, equipped with a Phenomenex Zebron ZB-5 ms (30 m × 0.25 mm, 0.25 μm) column (conditions as described above for the Trace MS, but in split-less mode). HRMS (ESI^-) for compound **10** was performed on a Bruker Daltonics — maXis Ultra High ResolutionTOF instrument.

NMR spectra were recorded at 300 K either on a Bruker DRX500 spectrometer (operating frequency 500 MHz for ^1H and 125 MHz for ^{13}C) or a Bruker Avance 400 NMR spectrometer (operating frequency 400 MHz for ^1H and 100 MHz for ^{13}C). ^1H NMR chemical shifts were referenced relative to the TMS signal or the residual solvent peak. As compounds are mostly mixture of isomers, MS and NMR data are reported for the major isomer only. For copy of more important spectroscopic data see reference [39].

4.2. Synthetic procedures

4.2.1. Methyl (Z)-2-(6-(pent-2-en-1-yl)-1,4-dioxaspiro[4.4]nonan-7-yl)acetate (*2*)

A 50 ml round-bottomed flask was charged with commercially available MeJA (**1**) (2.461 g, 11 mmol), 1,2-ethanediol (0.749 g, 12.1 mmol), dry C_6H_6 (10 ml), and *p*-TsOH (0.07 g, catalyst). The flask was attached to a Dean-Stark trap, refluxed for 4 h and worked up. The crude product **2** (6.118 g; 96.7%) was employed in the next reaction without purification. GC-MS (EI^+): *m*/*z*(%): 41.18(18), 55.03(32), 67.00(38), 85.94(51), 99.00(100), 153.07(64), 195.08(55), 268.22 $[M]^+$ (36).

4.2.2. (Z)-2-(6-(pent-2-en-1-yl)-1,4-dioxaspiro[4.4]nonan-7-yl)ethan-1-ol (*3*)

The synthesis was carried as follow. A 100 ml three-necked flask under Ar atmosphere was charged with $LiAlH_4$ (1.082 g, 28.5 mmol), dry Et_2O (45 ml) and **2** (6.118 g, 22.8 mmol) dissolved in dry Et_2O (10 ml) was added dropwise. After the addition was complete, the mixture was further stirred for 1.5 h. The reaction mixture was worked up and evaporation of solvents afforded crude **3** (4.687 g; 85%) which was sufficiently pure for further transformation. GC–MS (EI^+): *m*/*z*(%): 55.07(41), 99.20(100), 153.20(35), 195.27(47), 240.31 $[M]^+$ (30).

4.2.3. (Z)-2-(6-(pent-2-en-1-yl)-1,4-dioxaspiro[4.4]nonan-7-yl) acetaldehyde (*4*)

A 250 ml three-necked flask, equipped with a magnetic stirring bar and pressure-equalizing funnel, was purged with argon and charged with dry CH_2Cl_2 (80 ml), finely powdered PCC (11.780 g, 54.6 mmol, 1.5 equiv.), AcONa (0.440 g), and 15 g of 4 Å molecular sieves in powder. Compound **3** (8.760 g, 36.4 mmol) dissolved in CH_2Cl_2 (20 ml) was

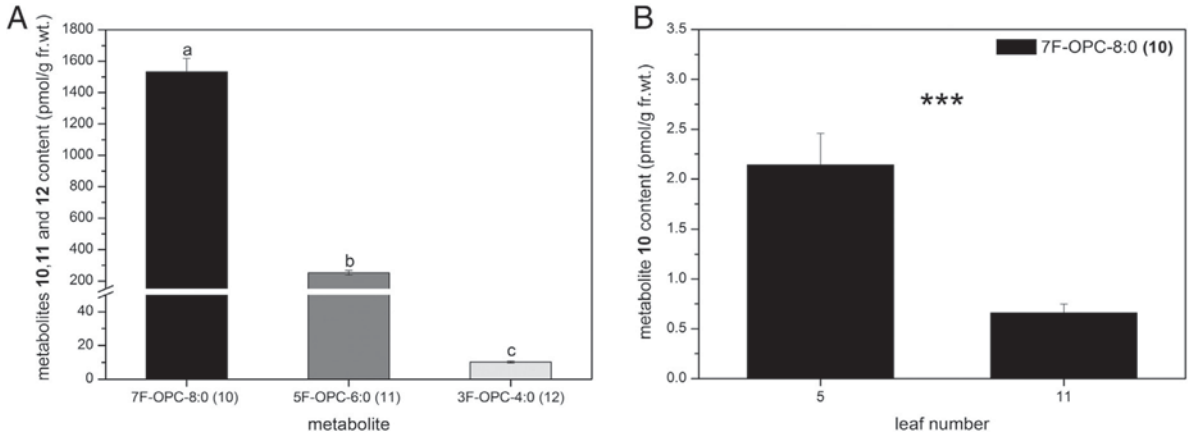

Fig. 6. Mean content (±s.e., n = 11) of 7F-OPC-8:0 (**10**), 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**) in *A. thaliana* Col-0 leaves after wounding and treatment with **10** for 60 min. (**A**) Contents of **10**, **11** and **12** in the treated local leaf 8. Statistically significant differences between the content of the metabolites were analyzed by One-Way ANOVA ($p < 0.05$, SNK test). (**B**) Content of **10** in systemically connected leaves 5 and 11. Metabolites **11** and **12** were not detected in leaves 5 and 11. Statistically significant differences between content of **10** in different leaves were analyzed by Mann–Whitney Rank Sum Test ($p < 0.05$, *** $p < 0.001$).

added dropwise to the reaction mixture which was stirred for 4 h (room temp.), and then filtered through a pad of Florisil. The filtrate was concentrated on a rotary evaporator, and the residual oil was purified by flash chromatography on silica gel (EtOAc/*n*-hexane, 1:4) to give **4** (6.270 g, 72%) as a colorless oil. GC–MS (EI^+): *m/z*(%): 55.12(48), 99.27(100), 153.43(31), 194.60(38), 195.61(45), 238.63 $[M]^+$ (8). ^1H NMR (500 MHz, $CDCl_3$): δ 9.72 (s, 1 H), 5.30–5.40 (m, 2 H), 3.82–3.93 (m, 4 H), 2.54–2.71 (m, 1 H), 2.35 (ddd, J = 16.7, 9.5, 2.4 Hz, 1 H), 2.12–2.26 (m, 2 H), 2.01–2.10 (m, 3 H), 1.92–1.99 (m, 1 H), 1.61–1.83 (m, 3 H), 1.19–1.33 (m, 1 H), 0.94 ppm (t, J = 7.6 Hz, 3 H); ^{13}C NMR ($CDCl_3$, 126 MHz): d = 202.2, 132.4, 127.5, 117.4, 64.7, 64.2, 51.3, 49.9, 37.1, 35.2, 28.1, 26.5, 20.5, 14.1 ppm.

4.2.4. 2-((6-iodohexyl)oxy)tetrahydro-2 H-pyran (**6**)

6-iodohexan-1-ol (**5**) was prepared as described in Ng and Fromherz [48]. Compound **5** was obtained as thick yellow oil (2.478 g, 98%) and directly employed in the next reaction. The tetrahydropyranyl ether of **5** was prepared by stirring a solution of **5** (2.478 g, 10.86 mmol) and 2,3-dihydropyran (4.579 g, 54.44 mmol; 5 equiv.) in CH_2Cl_2 (50 ml, room temp.) was added *p*-TsOH (0.025 g), the mixture stirred for 2 h, and then worked up. The remaining faintly yellow oil was chromatographed on silica gel (*n*-hexane-EtOAc, 9:1) to afford pure **6** (2.502 g, 74%). GC–MS (EI^+): *m/z*(%): 41.34(58), 55.03(99), 83.01(60), 84.74(100), 168.96(15), 311.10(20), 312.22 $[M]^+$ (4).

4.2.5. (Z)-1-(6-(pent-2-en-1-yl)-1,4-dioxaspiro[4.4]nonan-7-yl)-8-((tetrahydro-2 H-pyran-2-yl)oxy)octan-2-ol (**7**)

An oven-dried 50 ml flask was charged with **6** (0.500 g, 1.6 mmol) and dry *n*-pentane/Et_2O (16 ml, 3:2) under argon atmosphere to give an approximately 0.1 M solution. All additions were performed by using argon-flushed syringes and a positive pressure of argon was maintained within the flask during all subsequent operations. The flask was cooled to −78 °C with a dry ice–acetone bath and *t*-BuLi (2.2 ml, 1.6 M in *n*-pentane, ca. 2.2 equiv.) was then added dropwise via syringe. Stirring was continued at −78 °C for additional 5 min following the addition, the cooling bath was then removed, and the mixture was allowed to warm and stand at room temperature for 1 h to consume unreacted *t*-BuLi. Afterwards, aldehyde **4** (0.515 g, 1.5 equiv.) was added dropwise and the reaction mixture was worked up. Flash chromatography (*n*-hexane/Et_2O, 1:1) afforded **7** (0.305 g, 45%). GC–MS (EI^+): *m/z*(%): 41.20(15), 54.98(27), 85.02(100), 99.04(93), 153.03(14), 195.02(19), 239.18(5), 339.26(8), 424.35 $[M]^+$ (0.5).

4.2.6. (Z)-7-(2-fluoro-8-((tetrahydro-2H-pyran-2-yl)oxy)octyl)-6-(pent-2-en-1-yl)-1,4-dioxaspiro[4.4]nonane (**8**)

To a solution of DAST (0.090 ml, 0.65 mmol, 1.2 equiv.) in dry CH_2Cl_2 (0.26 ml) at −78 °C was added under Ar a solution of the alcohol **7** (0.207 g, 0.49 mmol) in dry CH_2Cl_2 (0.1 ml) via argon-flushed syringe. The solution was stirred at −78 °C for 2 h and 3 h after removal of the cooling bath. The reaction mixture was then quenched with saturated K_2CO_3 and the aqueous phase extracted with Et_2O (3 × 10 ml). The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude product **8** was submitted to deprotection without further purification.

4.2.7. (Z)-3-(2-fluoro-8-hydroxyoctyl)-2-(pent-2-en-1-yl)cyclopentan-1-one (**9**)

Deprotection of **8** was achieved in one step by stirring the compound in a mixture of Me_2CO/EtOH/water (1:1:1) containing PPTS (0.010 g, 10% of the alcohol). Flash chromatography on silica gel (Et_2O/*n*-pentane, 3:2) afforded the desired product **9** (0.055 g, 38%, two steps). TLC R_f 0.16. GC–MS (EI^+) **9**-TFA derivative: *m/z*(%): 40.68(30), 54.60(26%), 66.72(19), 82.81(100), 94.80(19), 108.82(19), 123.87(22), 151.00(23), 326.31(3), 394.34 $[M]^+$ (1). ^1H NMR (500 MHz, $CDCl_3$): δ5.30–5.43 (m, 1 H), 5.12–5.26 (m, 1 H), 3.63–3.71 (m, 1 H), 3.57 (t, J = 6.5 Hz, 2 H), 2.23–2.36 (m, 3 H), 2.06–2.23 (m, 2 H), 1.95–2.05 (m, 3 H), 1.73 (m, 2 H), 1.45–1.55 (m, 3 H), 1.22–1.44 (m, 10 H), 0.89 ppm (t, J = 7.5 Hz, 3 H); ^{13}C NMR (126 MHz, $CDCl_3$): δ 219.5, 132.6, 124.4, 68.3, 61.9, 54.1, 41.5, 37.5, 37.0, 36.7, 31.6, 28.4, 26.1, 24.7, 24.6, 24.3, 19.6, 13.2 ppm.

4.2.8. (Z)-7-fluoro-8-(3-oxo-2-(pent-2-en-1-yl)cyclopentyl)octanoic acid (7-F-OPC, **10**)

Jones reagent (4 M) was added to a solution of **9** (0.055 g, 0.18 mmol) in Me_2CO (2 ml) at 0 °C until the color of the reagent persisted. After 30 min at 0 °C, excess of the reagent was quenched by addition of 2-propanol. The resulting mixture was filtered through a pad of Celite by elution with Et_2O and washed several times with brine. The organic solution was dried over $MgSO_4$, concentrated under reduced pressure and the remaining oil purified by flash chromatography (CH_2Cl_2-Me_2CO, 1:1). 7-F-OPC (**10**) was obtained in 78% yield (0.044 g). HRMS (ESI^-TOF): *m/z* = 311.2042 [M–H] (calc. For $C_{18}H_{28}FO_3$, 311.2023) ^1H NMR (400 MHz, $CDCl_3$): δ5.36–5.54 (m, 1 H), 5.17–5.33 (m, 1 H), 4.44–4.74 (m, 1 H), 2.37 (m, 6 H), 1.95–2.19 (m, 5 H), 1.58–1.93 (m, 5 H), 1.30–1.56 (m, 6 H), 0.96 ppm (t, J = 7.5 Hz, 3 H); ^{13}C NMR ($CDCl_3$, 101 MHz): δ 213.3, 178.0, 133.9, 125.2, 92.5, 54.9, 40.2, 39.0, 38.1, 38.0, 35.6, 33.6, 28.9, 27.1, 25.5, 24.5, 20.6, 14.1 ppm.

4.3. Plant material and treatments

Plant treatments were carried out as described in reference. For investigating the systemic translocation of 7F-OPC-8:0 (**10**), the leaves of five-week old plants were numbered according to Farmer et al. [45]. Plants were wounded at leaf 8 with a pattern wheel parallel to the midrib as described [30]. A total amount of 20 μl of 50 μM 7F-OPC-8:0 (**10**) was applied to the wounds. Leaves 5, 8 and 11 of each plant were harvested 60 min after treatment.

4.4. RNA-isolation, RT-PCR and primers

For all details concerning gene induction analyses see reference [39].

4.5. Quantification of phytohormones and Arabidopsides

Analysis of phytohormones followed previously described methods with some modifications [30]. Finely ground leaf material (250 mg) was extracted with 1.5 ml of methanol containing 60 ng of [$^2H_6$]JA, and 12 ng of JA-[$^{13}C_6$]Ile conjugate as internal standards. The homogenate was mixed for 30 min and centrifuged at 13,000 rpm for 20 min at 4 °C and the supernatant was collected. The homogenate was re-extracted with 500 μl methanol, mixed and centrifuged and the supernatants were pooled. The combined extracts were evaporated under reduced pressure at 30 °C and dissolved in 500 μl methanol. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 × 4.6 mm, 1.8 μm, Agilent). Water and acetonitrile containing formic acid (0.05%) were employed as mobile phases A and B, respectively. The elution profile was: 0–0.5 min, 5% B; 0.5–9.5 min, 5–42% B; 9.5–9.51 min 42–100% B; 9.51–12 min 100% B and 12.1–15 min 5% B. The mobile phase flow rate was 1.1 ml min^{-1}. The column temperature was maintained at 25 °C. An API 5000 tandem mass spectrometer (Applied Biosystems) equipped with a Turbo Spray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards if available. The ion spray voltage was maintained at −4500 eV. The turbo gas temperature was set at 700 °C. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 7 psi. Multiple reaction monitoring (MRM) was used to monitor analyte parent ion → product ion: *m/z* 209.1 → 59.0 (CE −24 V; DP −35 V) for jasmonic acid; *m/z* 215.1 → 56.0 (CE −24 V; DP −35 V) for [$^2H_6$]JA; *m/z* 322.2 → 130.1 (CE −30 V; DP −50 V) for JA-Ile; *m/z* 328.2 → 136.1

(CE −30 V; DP −50 V) for JA-[$^{13}C_6$]Ile conjugate; and m/z 290.9 → 165.1 (CE −24 V; DP −45 V) for *cis*-OPDA. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies were verified by analyzing dilution series of standard mixtures. Phytohormones were quantified relative to the signal of their corresponding internal standard. For quantification of *cis*-OPDA, [$^2H_6$]JA was used as the internal standard applying an experimentally determined response factor of 0.5. Arabidopside A and Arabidopside B were determined as described [39].

4.6. Quantification of 7F-OPC-8:0 (**10**), 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**)

For the analysis of the fluorinated jasmonate 7F-OPC-8:0 (**10**) and its β-oxidation products, 5F-OPC-6:0 (**11**) and 3F-OPC-4:0 (**12**), the same extracts as for phytohormone quantification were used. In the systemic transport study, single leaf extraction was performed. The whole leaf material was used and extracted with 1 ml of MeOH containing 40 ng of [$^2H_6$]JA, and 8 ng of JA-[$^{13}C_6$]Ile conjugate as internal standards. Following the protocol mentioned above the combined, evaporated extract was dissolved in 200 μl MeOH. The following MRMs were added to the LC–MS/MS method described above: analyte parent ion → product ion: *m/z* 311.0 → 291.0 (collision energy (CE) − 20 V; declustering potential (DP) − 100 V) for 7F-PC-8:0 (**10**); *m/z* 283.0 → 263.0 (CE −20 V; DP −100 V) for 5F-OPC-6:0 (**11**); *m/z* 255.0 → 235.0 (CE −20 V; DP −100 V) for 3F-OPC-4:0 (**12**). For all four compounds, [$^2H_6$]JA was used as the internal standard applying a theoretical response factor of 0.5. The identity of compounds **11** and **12** was corroborated as described in [39].

Transparency document

The Transparency document associated with this article can be found, in online version.

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Supplementary information for article II. Reference [39] in article II.

Data article *(submitted to Data in Brief)*

Title: *Metabolism and chemical characterization of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0*

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Abstract

*Here we describe several physiological responses induced by a fluorinated mimic of the jasmonate precursor OPC-8:0 in Arabidopsis thaliana plants. In particular, we describe the induction of the jasmonate responsive genes (JRG) JAZ1, GST1 and OPR1 by the fluorinated compound 7F-OPC-8:0 (**10**). The (**10**)-dependent induced levels of JA-Ile, arabidopside A and arabidopside B are presented as well. Furthermore, we provide NMR and LC-MS characterization of compound **10** and some of its metabolic derivatives.*

Specifications Table

Subject area	*Chemistry and Biology*
More specific subject area	*Plant physiology*
Type of data	*Graph, figure, table*
How data was acquired	*RT-PCR: Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA). NMR: Bruker DRX500; Bruker Avance 400. Mass spectroscopy: Bruker Daltonics - maXis Ultra High ResolutionTOF; API 5000 tandem mass (Applied Biosystems); LTQ Orbitrap XL (Thermo Fisher Scientific, Bremen, Germany).*
Data format	*Analyzed*
Experimental factors	*RNA-isolation, phytohormone extraction,*
Experimental features	*RNA isolation: ca. 100 mg leaf material was homogenized and RNA extraction and cDNA synthesis as described in [11]. Phytohormone extraction: ca. 250 mg leaf material was extracted with 1.5 ml of methanol containing 60 ng of [$^{2}H_{6}$]JA, and 12 ng of JA-[$^{13}C_{6}$]Ile as internal standards [10].*
Data source location	*Jena, Germany.*
Data accessibility	*Data available with this article.*

Value of the data

- First physiological data for a fluorinated jasmonate compared to endogenous *cis*-OPDA.
- MS methods may be applicable for other fluorinated jasmonates
- Interesting profile for arabidopsides levels in fluorinated-jasmonate treated plants
- Useful NMR data of synthetic 7F-OPF-8:0

Data

Here we present how a fluorinated jasmonate precursor (7F-OPF-8:0) affects the expression of marker JRG *JAZ1*, *GST1* and *OPR1*. We characterize 7F-OPF-8:0 (**10**) and metabolic derivatives by LC-MS and quantify the levels of JA-Ile and arabidopsides A and B induced by **10**. Furthermore, we provide NMR spectra of this compound. In some cases data is directly compared to the endogenous jasmonate precursor *cis*-OPDA. Table S1 contains the list of primers employed for RT-PCR.

Experimental Design, Materials and Methods

Plant material and treatments: *Arabidopsis thaliana* ecotype Columbia was used for all experiments and plants were grown as previously described [10]. Four to five week old plants, grown under short-day conditions were sprayed with 0.75 ml (50 µM) of 7F-OPC-8:0 (**10**) or solvent control (0.125 % ethanol) and incubated for the indicated time periods. For *cis*-OPDA treatments, a solution of this compound (10 µM, containing 0.1% DMSO) was used. The solvent control was a 0.1% solution of DMSO. The *cis*-OPDA was synthesized according to the procedure previously described [85].

RNA-isolation and RT-PCR: For RNA isolation, 1 leaf (~ 100 mg) was harvested and stored in liquid nitrogen until use. Samples were homogenized with a Genogrinder 2010 (Spex Sample Prep, Stanmore, UK) for 1 min at 1000 rpm. RNA extraction and cDNA synthesis was performed as described before [11]. Q-RT-PCR was carried out in 96-well plates on a Bio-Rad CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, USA) by the use of Brilliant II QPCR SYBR green Mix (Agilent, Böblingen, Germany). Analysis of dissociation curve was performed for all primer pairs and *RPS18B* was used as endogenous control for all experiments. The obtained mRNA levels of the genes of interest were normalized to the *RPS18B* mRNA level in each cDNA probe. Expression levels were calculated by use of the normalized expression (ΔΔCq) in Bio-Rad CFX Manager Software (3.1). Untreated plants were used as control (expression level = 1). The primer pairs used are listed in supplementary materials (Table S1).

Quantification of JA-Ile, arabidopside A, arabidopside B, and MS data: Analysis of phytohormones followed previously described methods with some modifications [10]. Finely ground leaf material (250 mg) was extracted with 1.5 ml of methanol containing 60 ng of [$^{2}H_{6}$]JA, and 12 ng of JA-[$^{13}C_{6}$]Ile conjugate as internal standards. The homogenate was mixed for 30 min and centrifuged at 13000 rpm for 20 min at 4 ºC and the supernatant was collected. The homogenate was re-extracted with 500 µl methanol, mixed and

centrifuged and the supernatants were pooled. The combined extracts were evaporated under reduced pressure at 30 °C and dissolved in 500 µl methanol. Chromatography was performed on an Agilent 1200 HPLC system (Agilent Technologies). Separation was achieved on a Zorbax Eclipse XDB-C18 column (50 x 4.6 mm, 1.8 µm, Agilent). Water and acetonitrile containing formic acid (0.05%) were employed as mobile phases A and B respectively. The elution profile was: 0-0.5 min, 5% B; 0.5-9.5 min, 5-42% B; 9.5-9.51 min 42-100% B; 9.51-12 min 100% B and 12.1-15 min 5% B. The mobile phase flow rate was 1.1 ml min-1. The column temperature was maintained at 25 °C. An API 5000 tandem mass spectrometer (Applied Biosystems) equipped with a Turbospray ion source was operated in negative ionization mode. The instrument parameters were optimized by infusion experiments with pure standards if available. The ion spray voltage was maintained at -4500 eV. The turbo gas temperature was set at 700 ºC. Nebulizing gas was set at 60 psi, curtain gas at 25 psi, heating gas at 60 psi and collision gas at 7 psi. Multiple reaction monitoring (MRM) was used to monitor the analytes' parent ions → product ion: *m/z* 215.1 →56.0 (CE -24 V; DP -35 V) for [$^{2}H_{6}$]JA; *m/z* 322.2 →130.1 (CE -30V; DP -50V) for JA-Ile and *m/z* 328.2 →136.1 (CE -30V; DP -50V) for JA-[$^{13}C_{6}$]Ile conjugate. Both Q1 and Q3 quadrupoles were maintained at unit resolution. Analyst 1.5 software (Applied Biosystems) was used for data acquisition and processing. Linearity in ionization efficiencies were verified by analyzing dilution series of standard mixtures. Phytohormones were quantified relative to the signal of their corresponding internal standard.

For the quantification of arabidopside A and arabidopside B the same extract as for phytohormone analysis were used. Samples were analyzed by LC-MS/MS as described above with the following modifications: chromatographic gradient was: 0-0.5 min, 10% B; 0.5-4 min, 10-90% B; 4-7 min 90-100% B; 7-7.5 min 100% B and 7.5-10 min 10% B. The following MRMs were used: analytes' parent ions → product ion: *m/z* 773.5 →291.0 (collision energy (CE)-36 V; declustering potential (DP) -30 V) for arabidopside A; *m/z* 801.5 →291.0 (CE -36 V; DP -30 V) for arabidopside B. Relative quantification is presented as normalized peak area in relation to the internal standard [$^{2}H_{6}$]JA.

HRMS (ESI^{-}) for compound **10** was performed on a Bruker Daltonics - maXis Ultra High ResolutionTOF instrument by direct injection of a pure sample. The identity of compounds **11** and **12** was corroborated by LC-HRMS. MS analysis was carried out on a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). Measurement conditions: ESI negative ionization mode; capillary temperature 275 C, capillary voltage 35 V; full-scan mass spectra, mass range of *m/z* 100 – 1000; mass resolution of m/Δm 30000. The software XCALIBUR (Thermo Fisher Scientific, Waltham, MA, USA) was employed for data interpretation. LC was performed on UltraMate 3000 (Thermo Fisher Scientific, Bremen, Germany) equipment. Separation was achieved with an Acclaim RSLC C18 column (2.2µm, 2.1 x 150mm; Thermo Fisher Scientific, Bremen, Germany). Formic acid (0.1%) in water and acetonitrile were employed as mobile phases A and B respectively. The elution profile was: 0-15 min, 1-

100% B; 15-18 min, 100% B; 18-18.1 min 100-1% B; 18.1-24 min, 1% B. The mobile phase flow rate was 0.3 ml min^{-1}. The column temperature was maintained at 25 °C.

NMR acquisition: NMR spectra were recorded at 300K either on a Bruker DRX500 spectrometer (operating frequency 500 MHz for ^{1}H and 125 MHz for ^{13}C) or a Bruker Avance 400 NMR spectrometer (operating frequency 400 MHz for ^{1}H and 100 MHz for ^{13}C). ^{1}H NMR chemical shifts were referenced relative to the residual solvent peak $CDCl_3$. As compounds are mostly mixture of isomers, NMR data are reported for the major isomer only.

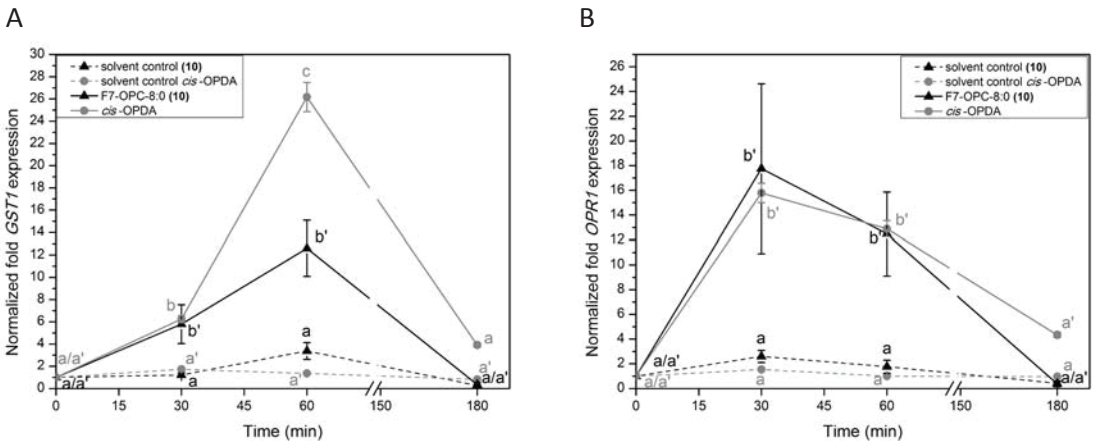


Fig. 1 Mean expression (± s.e., n=5) of *GST1* (A) and *OPR1* (B) in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**), *cis*-OPDA or the respective solvent control. Expression was analyzed after 30, 60 and 180 min. All samples were normalized to the *RPS18B* level and untreated plants were used as control. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

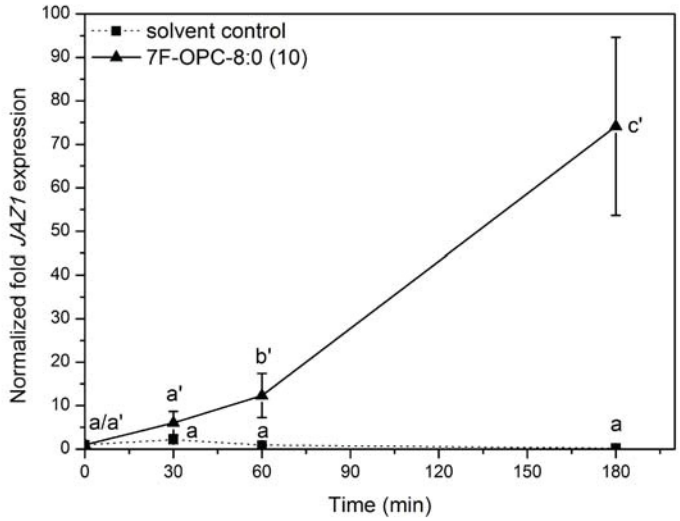


Fig. 2 Mean expression (± s.e., n=5) of *JAZ1* in *A. thaliana* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. Expression was analyzed after 30, 60 and 180 min. All samples were normalized to the *RPS18B* level and untreated plants were used as control. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

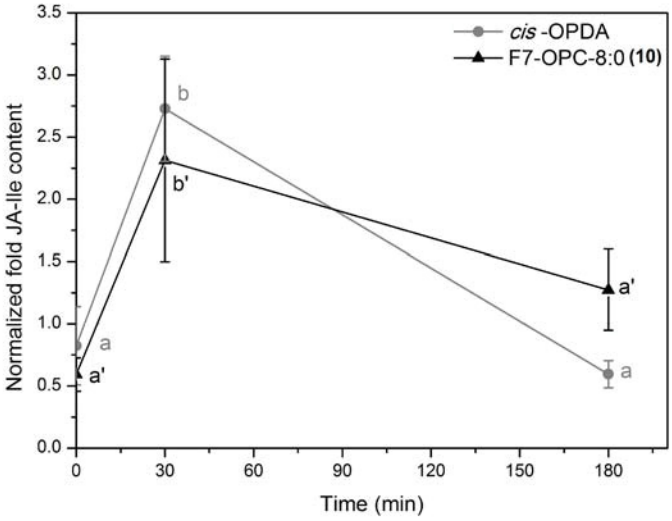


Fig. 3 Relative content (± s.e., n=5) of JA-Ile in *A. thaliana* Col-0 after treatment with *cis*-OPDA and 7F-OPC-8:0 (**10**). The content of JA-Ile was normalized to the respective solvent controls (level = 1). JA-Ile content was determined after 0 (untreated plants), 30, 180 min. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

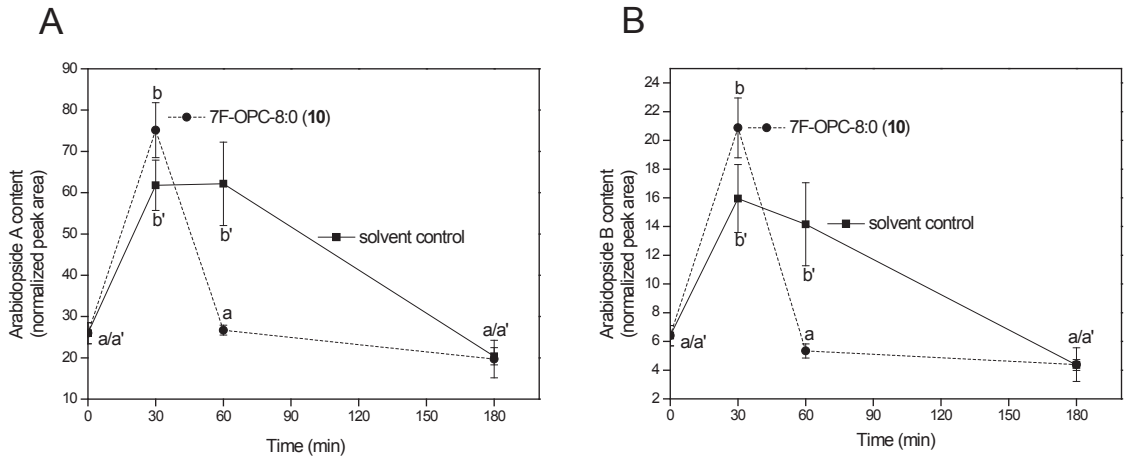


Fig. 4 Mean relative content (± s.e., n=5) of arabidopside A (A) and arabidopside B (B) in *Arabidopsis* Col-0 after treatment with 7F-OPC-8:0 (**10**) or solvent control. Measurements at 30, 60 and 180 min. Peak area was normalized to the IS $[^2H]_6$JA. Statistically significant differences were determined between the time points of the same treatment and were analyzed by One-Way ANOVA ($p < 0.05$, SNK test).

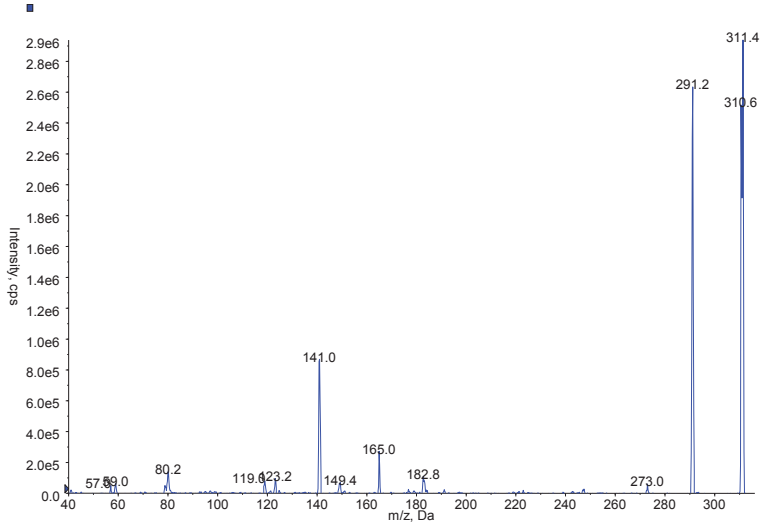


Fig. 5 MS2 spectrum of 7F-OPC-8:0 **(10)**. The fragmentation pattern of **10** reveals the molecular base peak $[M-H]^-$ (311.4 *m/z*) and a peak produced by the loss of HF ($[M-H-20]^-$, 291.2 *m/z*) as the most abundant fragments.

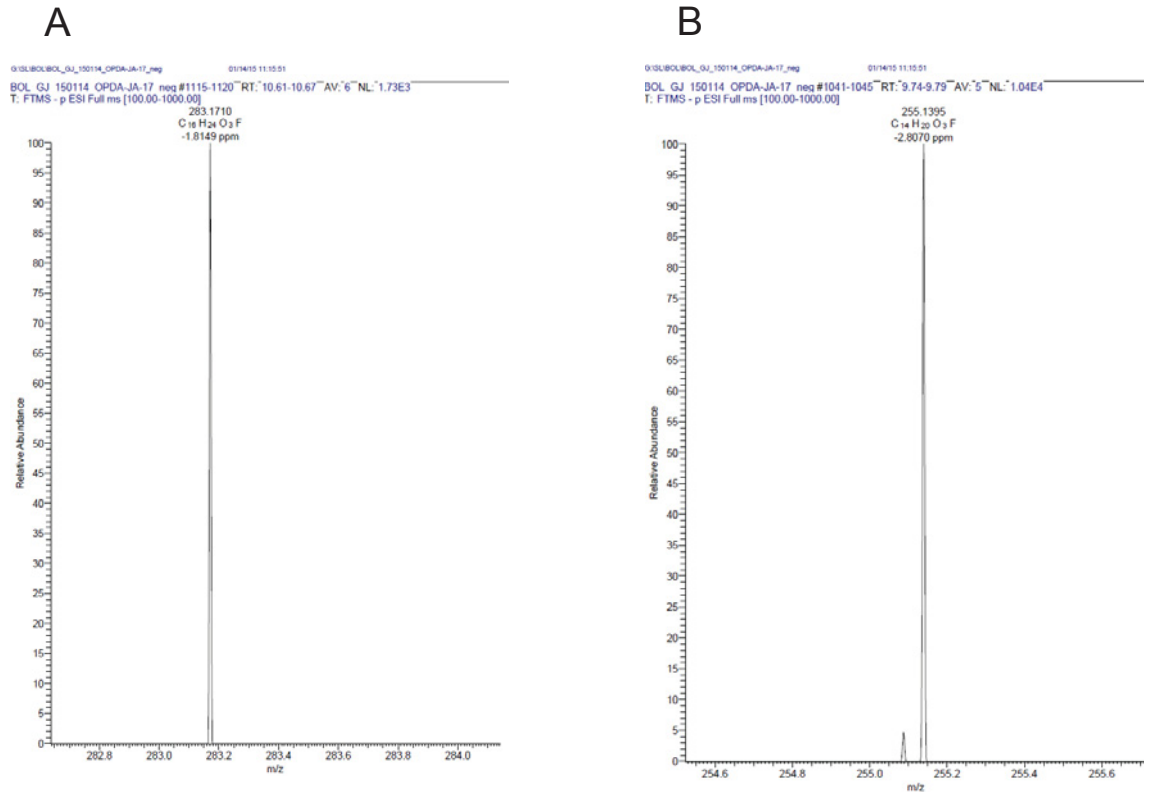
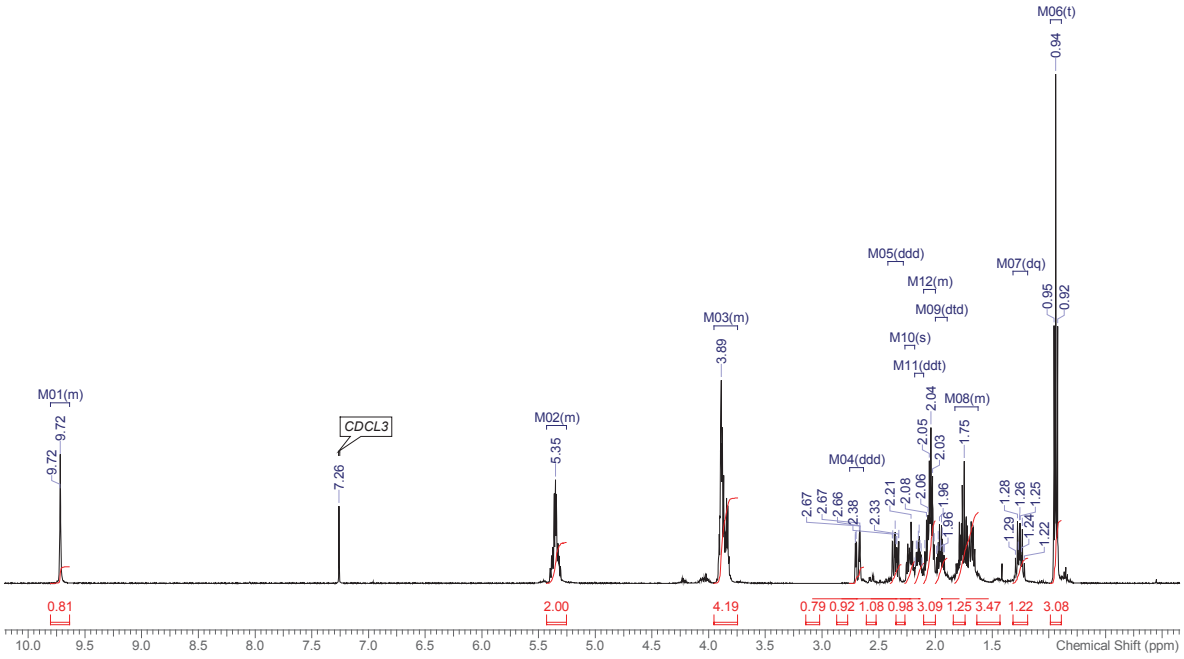


Fig. 6 HRMS spectra of compounds 5F-OPC-6:0 (**11**) (A) and 3F-OPC-4:0 (**12**) (B).

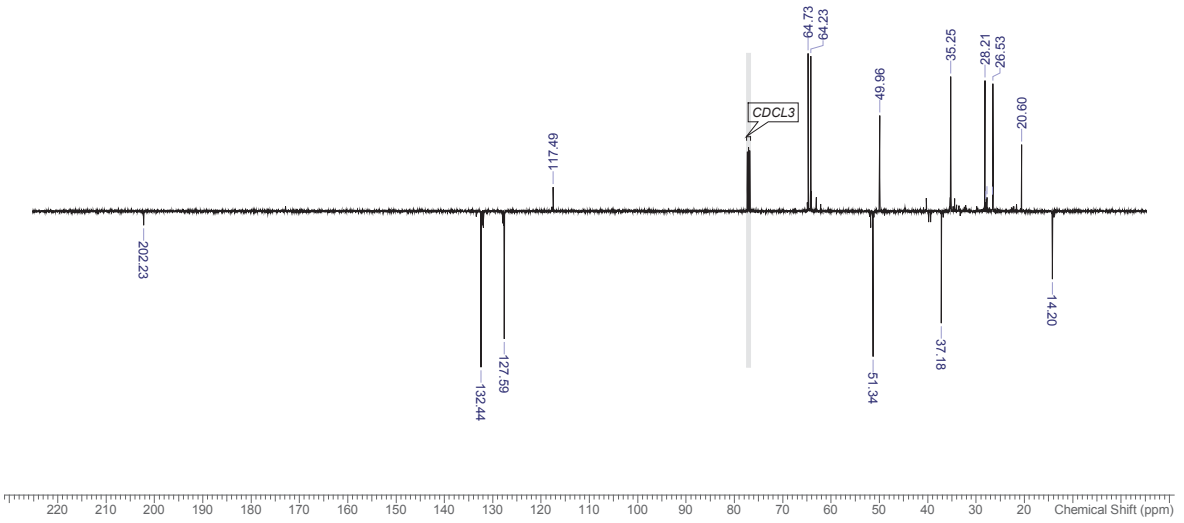
Table 1. Primers used for RT-PCR.

Target (Atg number)	Sequence
***RPS18B* (*At1g 34030*)**	5'- GTCTCCAATGCCCTTGACAT -3'
	5'- TCTTTCCTCTGCGACCAGTT -3'
OPR1 (At1g 76680)	5′- TGTGTCCTTGTTGTTGCAGGTTTTG -3′
	5′- TCCAACACGGTCTGGTCCGA -3′
OPR3 (At2g 06050)	5′- CCTTCTTCCAGATCGGCGGAGACAT -3'
	5′- GGCGCCAGAACCACTCGATGA -3'
GST1 (At1g 02930)	5'- GCCTTTCATCCTTCGCAACCCCT -3'
	5'- TCGCCATGTCCTTGCCAGTTGA -3'
VSP2 (At5g24770)	5'- ACGACTCCAAAACCGTGTGCAA -3'
	5'- CGGGTCGGTCTTCTCTGTTCCGT -3'
JAZ1 (AT1G19180)	5'- CGCGAGCAAAGGCACCGCTA -3'
	5'- TCCAAGAACCGGTGAAGTGAAGC -3'

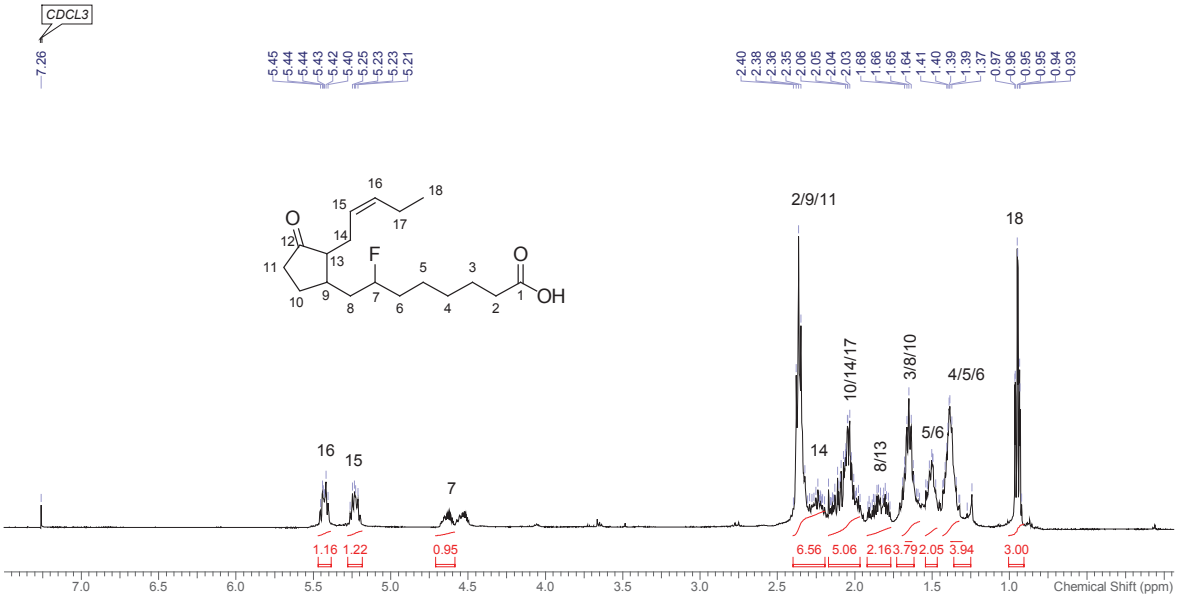
^{1}H-NMR Compound (**4**)



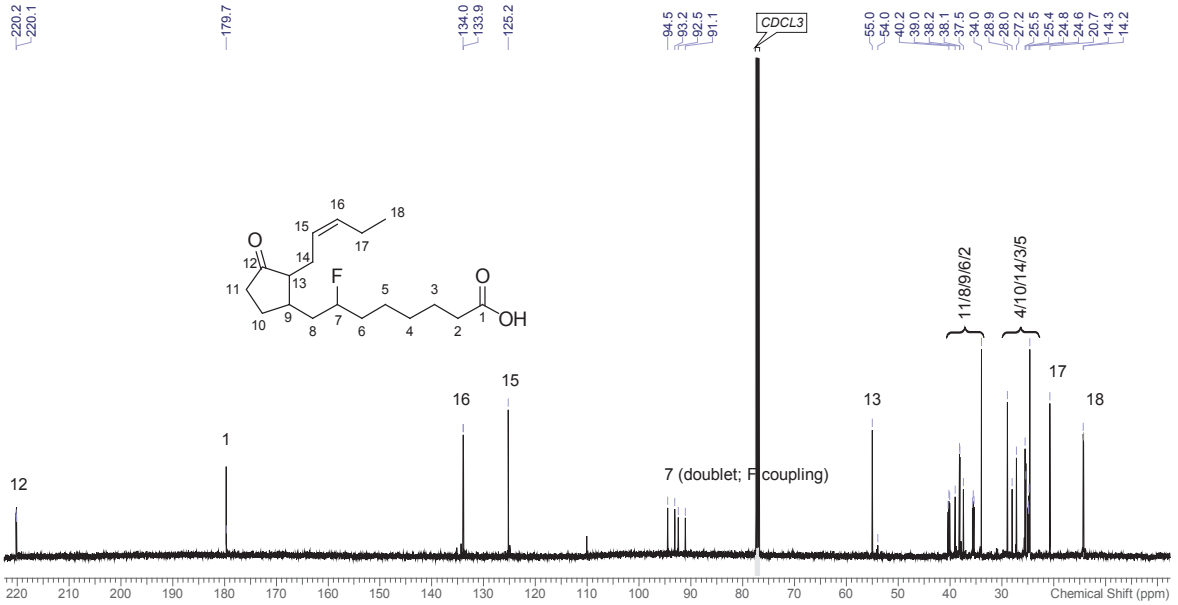
^{13}C- APT-NMR Compound (**4**)



^{1}H-NMR 7F-OPC-8:0 (**10**)



^{13}C NMR 7F-OPC-8:0 (**10**)



HRMS spectrum of 7F-OPC-8:0 (**10**)

Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\kepl2401\Guille_7fluoro8_cyclopentyloctanioicAcid_312neg_neuCalib.d	Acquisition Dat	12/5/2014 10:24:55 AM
Method	kp_tune_low_neg_250_JA.m	Operator	Admin
Sample Name		Instrument / Se	maXis 10078
Comment			

Acquisition Paramet

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	3000 V	Set Dry Heater	190 °C
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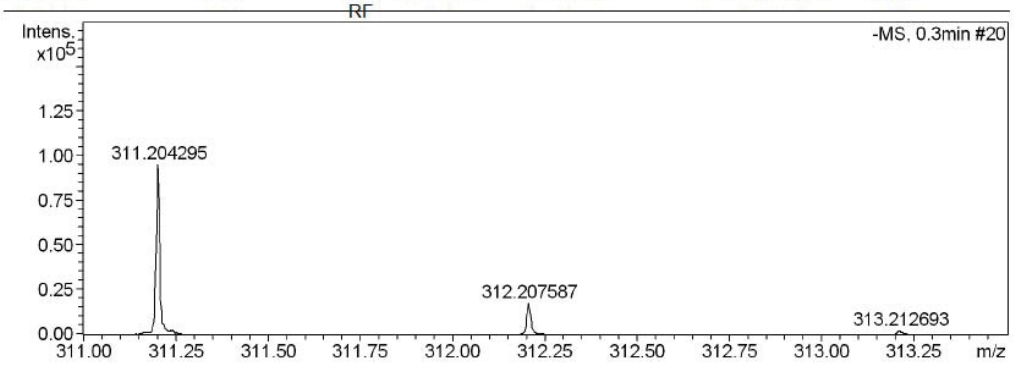
Scan End	1500 m/z	Set Collision Cell RF	1000.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
311.204295	1	C 18 H 28 F O 3	100.00	311.202797	-1.498	-4.814	5.6	4.5	even	ok

Acknowledgements

Funding granted by the Max Planck Society is gratefully acknowledged. We thank Sybille Lorenz and Kerstin Ploß for the HRMS measurements.

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6. Article III

Synthesis, structural characterization and biological activity of two diastereomeric JA-Ile macrolactones

Guillermo H. Jimenez-Aleman, Ricardo A. R. Machado, Helmar Görls, Ian T. Baldwin, and Wilhelm Boland

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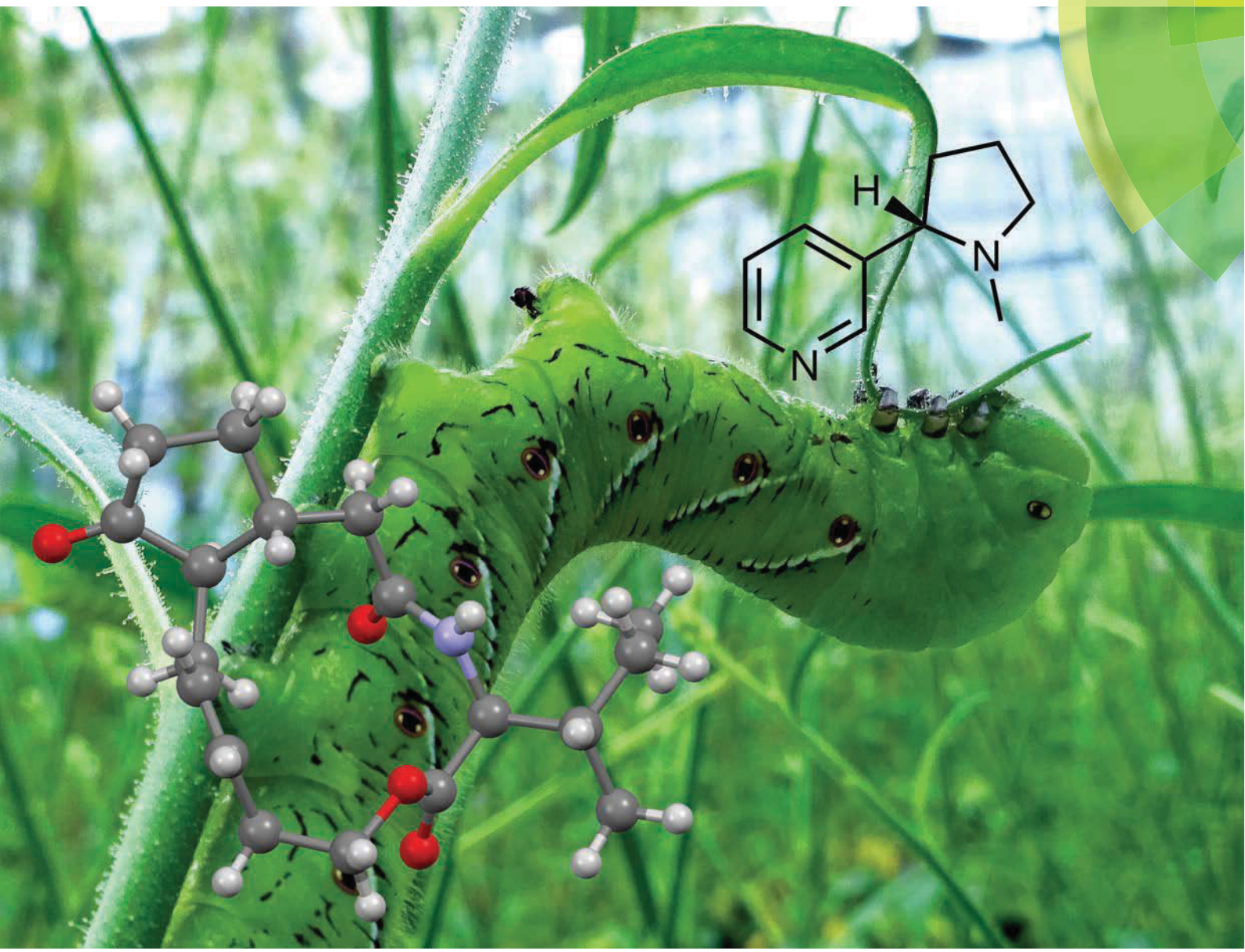

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PAPER
Wilhelm Boland *et al.*
Synthesis, structural characterization and biological activity of two diastereomeric JA-Ile macrolactones

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PAPER

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Synthesis, structural characterization and biological activity of two diastereomeric JA-Ile macrolactones†

Guillermo H. Jimenez-Aleman,[a] Ricardo A. R. Machado,[b] Helmar Görls,[c] Ian T. Baldwin[b] and Wilhelm Boland*[a]

Jasmonates are phytohormones involved in a wide range of plant processes, including growth, development, senescence, and defense. Jasmonoyl-L-isoleucine (JA-Ile, **2**), an amino acid conjugate of jasmonic acid (JA, **1**), has been identified as a bioactive endogenous jasmonate. However, JA-Ile (**2**) analogues trigger different responses in the plant. ω-Hydroxylation of the pentenyl side chain leads to the inactive 12-OH-JA-Ile (**3**) acting as a "stop" signal. On the other hand, a lactone derivative of 12-OH-JA (**5**) (jasmine ketolactone, JKL) occurs in nature, although with no known biological function. Inspired by the chemical structure of JKL (**6**) and in order to further explore the potential biological activities of 12-modified JA-Ile derivatives, we synthesized two macrolactones (JA-Ile-lactones (**4a**) and (**4b**)) derived from 12-OH-JA-Ile (**3**). The biological activity of (**4a**) and (**4b**) was tested for their ability to elicit nicotine production, a well-known jasmonate dependent secondary metabolite. Both macrolactones showed strong biological activity, inducing nicotine accumulation to a similar extent as methyl jasmonate does in *Nicotiana attenuata* leaves. Surprisingly, the highest nicotine contents were found in plants treated with the JA-Ile-lactone (**4b**), which has (3*S*,7*S*) configuration at the cyclopentanone not known from natural jasmonates. Macrolactone (**4a**) is a valuable standard to explore for its occurrence in nature.

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Introduction

Jasmonates (JAs) are a large family of lipid-derived plant metabolites that mediate responses to stress and regulate development.[1,2] These compounds owe their name to the initial isolation and characterization of methyl jasmonate (MeJA, **7**) from jasmine oil *Jasminum grandiflorum* in the early 1960s.[3] Since then, many JAs have been detected and isolated from different plant species.[4] JAs were first studied because of their properties as odorants, greatly appreciated in perfumery,[5] later – and more important – because of their role as phytohormones.[6–11] JAs occur throughout the plant kingdom (algae, mosses, gymnosperms and angiosperms) and also in fungi. The capacity to produce or transform JAs is extraordinarily high in fungi.[12] JA (**1**) is one of the key players of the JA family. It is biosynthesized by consecutive enzymatic reactions starting from linolenic acid (Fig. 1).[13] JA (**1**) is produced as the *cis* isomer (with respect to the cyclopentanone ring) (3*R*,7*S*)-JA, but it can readily epimerize at C7 to the more stable *trans* form (3*R*,7*R*)-JA. Both isomers exist in equilibrium in the cell and have different biological activities; generally, the *cis* isomer is more active although it is often found in lower amounts.[14–18] Several enzymes can act on JA (**1**) and transform it into numerous derivatives (Fig. 1). One of these metabolites is 12-OH-JA (**5**) which has been described as a potent tuber-inducing agent.[19,20] The hydroxy acid (**5**) is believed to be the natural precursor of JKL (**6**), a naturally occurring 10-membered ring macrolactone. JKL (**6**) was the first jasmonate to be reported in the literature, interestingly only in its *trans* form and with no biological activity known to date.[21–25]

JA-Ile (**2**), an amino acid conjugate of JA (**1**), is a bioactive endogenous jasmonate. This was postulated in 1995 by Krumm *et al.*[26] and later confirmed by the discovery that, in *A. thaliana*, JAR1 activates JA by conjugation with L-isoleucine.[18,27] Accumulation of JA-Ile (**2**) is observed in different plant tissues in response to environmental stresses,[27] but

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†Electronic supplementary information (ESI) available: Fig. S1, S2 and a copy of NMR spectra of important compounds. CCDC 1004515 and 1004516. For ESI and crystallographic data in CIF or other electronic format see DOI: 10.1039/c5ob00362h

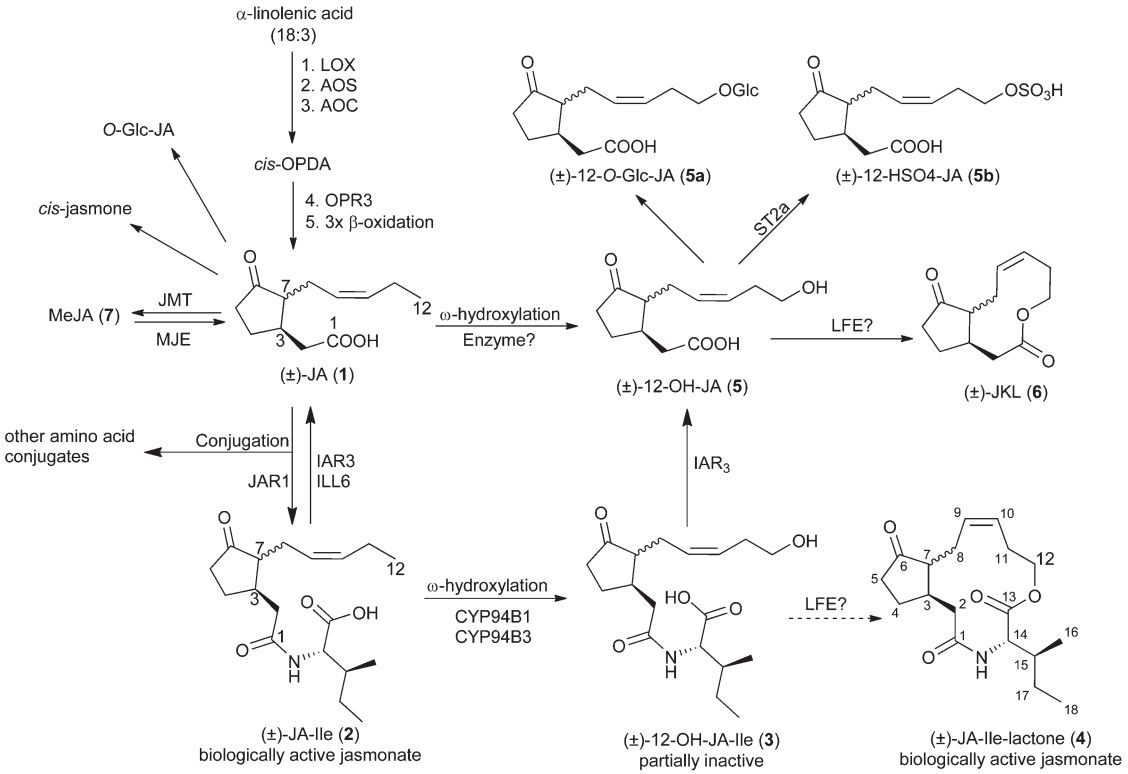

Fig. 1 Simplified scheme of the biosynthesis of jasmonates. JA (**1**) biosynthesis starts with the release of linolenic acid in the plastid. The succession of LOX, AOS, and AOC catalyzes the formation of OPDA, which is transformed by the action of OPR and three rounds of β-oxidations into JA (**1**). Hydroxylation of (**1**) produces 12-OH-JA (**5**), which is the precursor of (**5a**), (**5b**) and possibly JKL (**6**). The activation of JA (**1**) is catalyzed by JAR1 that conjugates it to L-Ile and generates JA-Ile (**2**). Turnover of (**2**) is carried out by members of the CYP94 family producing the hydroxy acid (**3**), a likely precursor of JA-Ile-lactone (**4**). The compounds are shown in the (3,7)-*cis*/*trans* stereochemistry occurring *in planta*. LOX, 13-lipoxygenase; AOS, 13-allene oxide synthase; AOC, allene oxide cyclase; OPR, 12-oxophytodienoate reductase 3; OPDA, *cis*-(+)-12-oxo-phytodienoic acid; JMT, JA carboxyl methyltransferase; JME, JA methylesterase; JAR1, JA-amino synthetase; ILL6, IAR3: JA-Ile amidohydrolases; CYP94B1/B3, cytochromes P450; LFE, lactone forming enzyme. The dashed arrow indicates a proposed transformation. Nomenclature and numbering commonly used for jasmonates was employed for clarity.

when the activating signal is no longer needed, JA-Ile (**2**) is converted to 12-OH-JA-Ile (**3**) by hydroxylation at C12.[28] The structure of (**3**) possesses a free carboxylic acid group and a hydroxyl group in analogy to the molecule of 12-OH-JA (**5**). Since JKL (**6**) exists in nature, a macrolactone like JA-Ile-lactone (**4**), derived from 12-OH-JA-Ile (**3**), may also exist (Fig. 1). Moreover, JA-Ile (**2**) analogues have shown different biological activities. For instance, the phytotoxin coronatine (Fig. S1, ESI†), a structural mimic of JA-Ile (**2**), is considerably more active than (**2**) in promoting the interaction of the COI1 (coronatine-insensitive 1) receptor with the JAZ (jasmonate ZIM domain) repressors *in vitro*.[29] The stronger activity of coronatine compared with JA-Ile (**2**) can be explained by two reasons: (i) the larger surface area provided by the cyclohexene ring compared with the corresponding area of the pentenyl side chain of JA-Ile (**2**) to interact with the COI1 receptor,[30] and (ii) the high stability of the *cis*-hydrindanone moiety of coronatine.[31] The methyl oxime derivatives of JA-Ile (**2**) and coronatine (Fig. S1, ESI†) act as JA-perception antagonists by binding to the COI1 receptor and hindering the interaction with the JAZ repressors due to the oxime group.[32] As the idea of tailoring jasmonate analogues for specific applications has been discussed,[33] these findings suggest a means of manipulating the JA-signaling pathway by chemically modifying the ligand (JA-Ile, **2**).

We hypothesized that a lactone such as the JA-Ile-lactone (**4**) analogous to JKL (**6**) may exist in nature. Furthermore, such a lactone retains the important moieties required for jasmonate perception and therefore may be biologically active.[16] Herein, we present a brief and efficient synthesis to JA-Ile-lactone (**4**). The biological activity of this new synthetic jasmonate was evaluated together with its diastereoisomer (**4b**). Since JKL (**6**) has been only described in the *trans* form, we prepared the (3*R*,7*R*)-isomer of the lactone (**4a**).

Results and discussion

Synthesis of the JA-Ile-lactone (4)

Our synthetic approach is based on two previously reported studies of jasmonates.[34,35] This route allows not only the synthesis of JA-Ile-lactone (**4**), but also the preparation of other 12-modified jasmonates which are of great biological interest

(*e.g.*, compounds (**5**), (**5a**), (**5b**) and (**3**)).[36] Furthermore, this synthetic route provides the opportunity to prepare enantiomerically pure amino acid conjugates of JA (**1**) starting from a racemic mixture of commercially available MeJA (**7**).[35] Although most of the bioassays were carried out with commercially available or synthetic JAs consisting of mixtures of isomers, inhibition by the non-natural isomers has not been reported to date.[33,37–40]

The synthesis of JA-Ile-lactone (**4**) starts from MeJA (**7**) as depicted in the retrosynthetic analysis (Fig. 2). Key steps are the Wittig reaction to generate the *cis* olefin (**9**) (Fig. 3, step b) and the final macrolactonization to JA-Ile-lactone (**4**). Ozonolysis of (**7**) as previously reported (CH_2Cl_2–MeOH, 1 : 1)[34] produced a mixture of the aldehyde (**8**) and the dimethyl acetal. To avoid acetal formation, the reaction was conducted in CH_2Cl_2, and the aldehyde (**8**) was obtained in 94% yield after flash chromatography. A regioselective Wittig reaction of the aldehyde (**8**) with the Wittig reagent (**15**)‡ directly afforded the keto ester (**9**). The conditions employed in the reaction produced the *cis* isomer of (**9**) with high selectivity (>95%, estimated by ^{1}H and ^{13}C NMR, ESI†) and yield (70%). This is a reliable procedure to obtain 12-modified jasmonates enriched with the *Z*-isomer. Long synthetic routes to these important metabolites are no longer needed.[41] Saponification of (**9**) afforded the free acid (**10**) quantitatively, which was employed in the next step without purification. Conjugation of L-Ile to (**10**) was carried out according to the procedure described for the linolenic acid/L-Ile conjugate.[42] This procedure was more straightforward than the procedure reported by Kramell *et al.*[35] The amino acid conjugate (**11**) (86% crude yield) was directly deprotected with *p*-TsOH in EtOH to afford the seco-acid 12-OH-JA-Ile (**3**) in 90% yield.

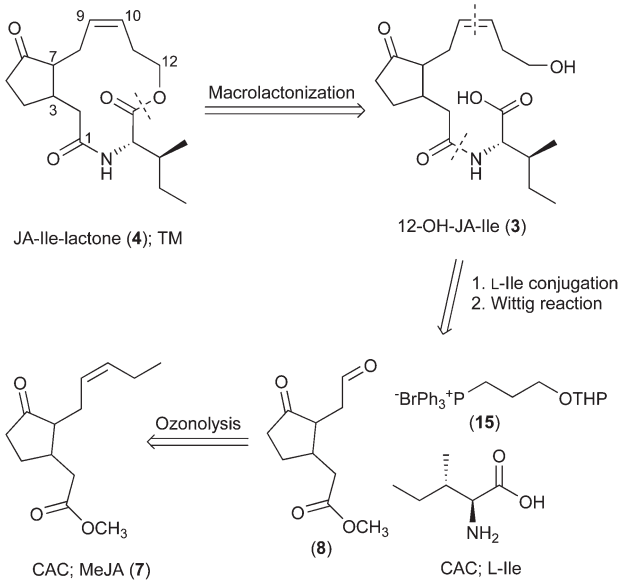

Fig. 2 Retrosynthetic analysis of JA-Ile-lactone (**4**). TM, target molecule; CAC, commercially available compound.

Macrolactonization to JA-Ile-lactone (**4**) was the most challenging step of the synthesis. Classical Yamaguchi–Yonemitsu conditions (Et_3N, $Cl_3C_6H_2COCl$, PhMe or PhH, DMAP) did not work at all, but we obtained excellent yields of the macrolactone (**4**) employing the two-step sequential reaction described by Ohba *et al.*[43] Using the ethoxyvinyl-ester (EVE) method to activate the acid group of (**3**), the macrolactone (**4**) was obtained (64% total yield) after flash chromatography. The JA-Ile-lactone (**4**) (mixture of isomers) was chromatographed on silica gel with AcOEt–*n*-hexane (7 : 3) and afforded two major products, JA-Ile-lactone (**4a**) (11.6 mg, 46%, TLC R_f = 0.26) and JA-Ile-lactone (**4b**) (13.7 mg, 54%, TLC R_f = 0.19). Finally, recrystallization from AcOEt–*n*-hexane and from EtOH–acetone afforded diastereomerically pure JA-Ile-lactones (**4a**) and (**4b**) respectively, as determined by NMR (ESI†).

Crystallography and structural characterization

Lactones (**4a**) and (**4b**) crystallize as orthorhombic colorless prisms. Both are packed in chain-like structures with the proton of the N–H group hydrogen bonding with the keto-amide group of the adjacent molecule (Fig. S2, ESI†). This orientation differs from the packing observed for indanoyl-isoleucine derivatives, which crystallize as dimers (sandwich-like) with two hydrogen bonds involving the keto group of the cyclopentanone ring and the N–H protons.[44] The absolute configuration of JA-Ile-lactone (**4a**) was assigned by reference to a chiral center of the L-Ile moiety (C14, Fig. 4). The absolute configuration of JA-Ile-lactone (**4b**) was additionally confirmed by anomalous-dispersion effects in diffraction measurements on the crystal using the intensity quotients method.[45]

JA-Ile-lactones (4a) and (4b) induce nicotine biosynthesis

Naturally occurring and synthetic JA analogs may have diverse biological backgrounds and activities.[6,7,46–48] These facts have made JAs the target of several synthetic studies that examined the relationship between the molecular structure and their activity.[7,16,49] Inspired by the structure of JKL (**6**) and other JA-Ile analogues, like coronatine, we designed and synthesized JA-Ile-lactones (**4a**) and (**4b**). The structures of these lactones possess all moieties known to be necessary for the bioactivity of JAs (*e.g.*, pentenyl side chain on C7, cyclopentanone ring, L-Ile moiety).[30] In addition, the macrocycle may confer a certain rigidity to the structure, in analogy to the cyclohexene ring to the molecule of coronatine or the aromatic ring in coronalon (Fig. S1, ESI†).

Nicotine is a typical direct defense stimulated by JAs in tobacco plants.[50,51] When MeJA (**7**) is applied to the leaves it is rapidly converted into bioactive JAs that induce the accumulation of nicotine in *N. attenuata* plants and served as a positive control in our experiments.[52,53] To test the potential

‡The THP moiety was unstable under classical conditions (Ph_3P, toluene, and reflux). Acetonitrile as a solvent in the presence of K_2CO_3 avoided this problem. This protecting group is crucial to selectively obtain the *Z*-isomer in the Wittig reaction.

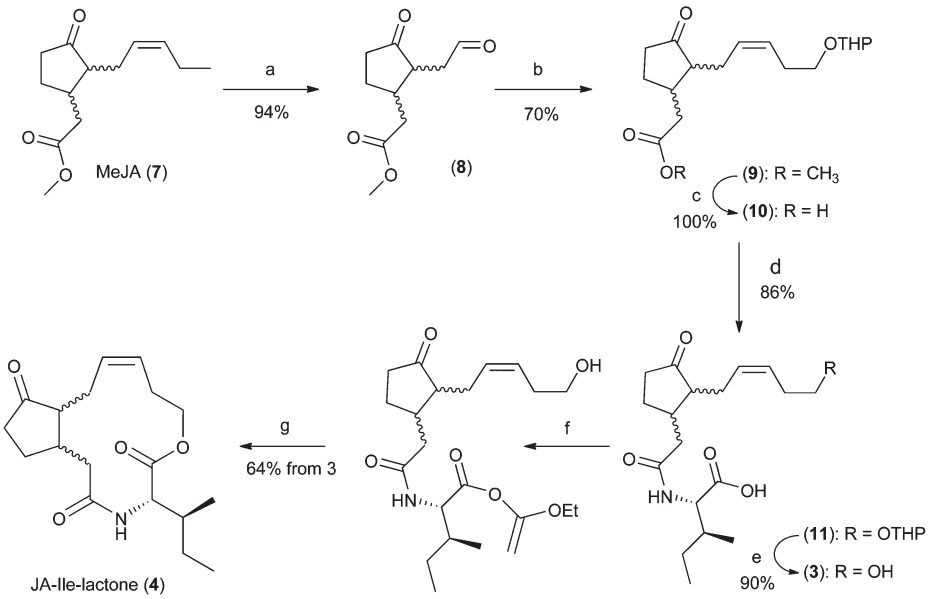

Fig. 3 Synthesis of JA-Ile-lactone (**4**). Reagents and conditions: (a) O_3, CH_2Cl_2, −78 °C, then $(Me)_2S$; (b) **15** (~0.2 M), KHMDS (1.2 equiv.), THF, −78 °C, then **8**, −78 °C to room temperature; (c) NaOH (~0.3 M), MeOH, 70 °C; (d) ethyl chloroformate ($ClCOOC_2H_5$, 1.1 equiv.), TEA, THF, L-Ile (2 equiv.), 0 °C; (e) pyridinium *p*-toluenesulfonate (10 mol%), EtOH, 55 °C; (f) ethoxyacetylene (4 equiv.), $[Ru(p\text{-cymene})Cl_2]_2$, acetone, 0 °C; (g) *p*-TsOH (0.05 M), 1,2-dichloroethane, high dilution, 50 °C.

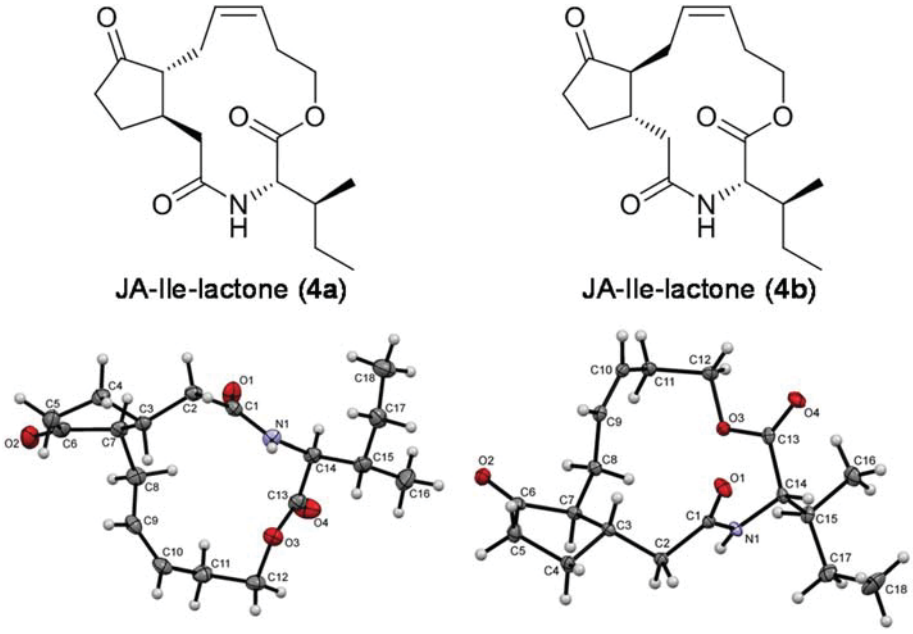

Fig. 4 Absolute configuration and crystal structures of lactones (**4a**) (3*R*,7*R*) – naturally occurring configuration, and (**4b**) (3*S*,7*S*) – not present in nature.

biological activity of the JA-Ile-lactones (**4a**) and (**4b**), we determined their ability to induce nicotine production in *N. attenuata* plants.

Both lactones induced nicotine accumulation in *N. attenuata* leaves similarly to MeJA (7) (Fig. 5). Strikingly, the JA-Ile-lactone (**4b**) induced the highest nicotine content although this molecule has a non-natural configuration at C3. To our knowledge, this is the first time that a jasmonate having the (3*S*,7*S*) configuration (not present *in planta*) is reported to strongly induce a secondary metabolite.

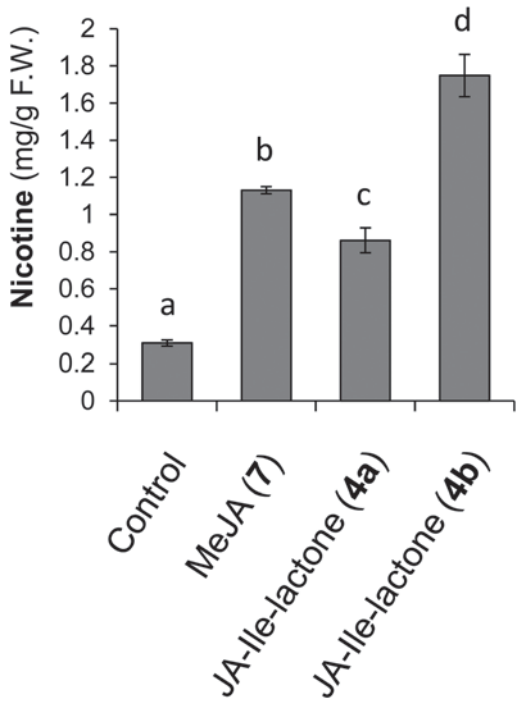

Fig. 5 Nicotine pools in leaves of *N. attenuata* plants after treatment (mean ± SEM) in milligrams per gram of fresh weight (mg g^{-1} F.W.). Control plants were treated with lanolin paste (n = 6), $P \leq 0.001$.

The precursor of the JA-Ile-lactones, the hydroxy acid 12-OH-JA-Ile (**3**), is a well-known jasmonate that acts as a stop signal in JA-signaling.[8,28,54] However, the lactones (**4a**) and (**4b**) derived from (**3**) are strongly active at inducing nicotine production. These results suggest that, similarly to JA-Ile (**2**), the JA-Ile-lactones may activate nicotine production *via* a COI1-dependent mechanism. Further studies are needed to test this hypothesis.

Conclusions

We have developed a short (7-step) and efficient synthesis (33% overall yield) of JA-Ile-lactones (**4**) from commercially available MeJA (**7**). A mixture of the synthesized JA-Ile-lactone (**4**) can be chromatographically resolved into the diastereomerically pure lactones (**4a**) and (**4b**). Furthermore, enantiomerically enriched C12-modified JA and JA-Ile derivatives (*e.g.*, **5a** and **5b**) can be prepared following our procedure. Both lactones are potent inducers of nicotine accumulation in the leaves of *N. attenuata* plants. The presence of such compounds in nature can now be explored with the synthetic JA-Ile-lactone (**4a**) as a reference. Furthermore, the rigid structure of these lactones makes them valuable molecules (templates) to study their interaction with the jasmonate receptor complex COI1/JAZ.[30,47] Understanding the mechanism of action of these new synthetic jasmonates will shed light on the JA-signaling pathway and therefore on plant–insect herbivore interactions.

Experimental section

General material and methods

All chemicals were obtained from commercial suppliers. If necessary, solvents were purified prior to use.[55] All work-up and purification procedures were carried out with reagent grade solvents. Thin layer chromatography was performed on silica gel 60 F^{254} on aluminum plates (Merck) and visualized with potassium permanganate staining or phosphomolybdic acid in ethanol. Melting points of the lactones were measured in capillary tubes on a Büchi B-540 instrument and are uncorrected. Flash chromatography was performed on silica gel 60 (40–63 μm) from Merck. Proportions of the employed solvents are referred to volume (v/v) otherwise mentioned.

GC-MS spectra were recorded on a ThermoQuest CE Instruments GC 2000 Series coupled to a ThermoQuest Finnigan Trace MS mass spectrometer. GC column: HP-5MS capillary column (15 m × 0.25 mm ID with 0.25 μm film thickness, Phenomenex). Injection port: 250 °C; flow, 15 mL min^{-1} with a split ratio of 10 mL min^{-1}; temperature programme: 60 °C (2 min) at 15 °C min^{-1} to 280 °C (5 min). Helium at 1.5 mL min^{-1} served as a carrier gas. The ionization method was electron impact (70 eV) in positive mode (EI^+). HRMS (ESI^+) was performed on a Bruker Daltonics maXis Ultra High Resolution TOF equipment.

NMR spectra were recorded at 300 K either on a Bruker DRX500 spectrometer (operating frequency 500 MHz for ^1H NMR and 125 MHz for ^{13}C NMR) or a Bruker Avance 400 NMR spectrometer (operating frequency 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR). ^1H NMR chemical shifts were referenced to TMS. Ozone was generated with a Sander Labor-Ozonisator, oxygen flow at 120 L h^{-1}, 0.44 A, 90% power at 20 °C.

Synthetic procedures

Synthesis of methyl 2-(3-oxo-2-(2-oxoethyl)cyclopentyl)-acetate (8). Ozone was bubbled into a solution of methyl jasmonate (MeJA, 3.08 g, 13.7 mmol) in CH_2Cl_2 (150 mL) at −78 °C until the blue color indicated an excess of ozone. A current of nitrogen was passed through the solution to eliminate the excess of ozone, $(Me)_2S$ (5 mL, 68.2 mmol, *ca.* 5 equiv.) was added and the mixture was stirred overnight and allowed to come to room temperature (RT). The mixture was then concentrated under reduced pressure and chromatographed on silica gel (*n*-hexane–AcOEt, 2 : 1) to afford (**8**) (2.56 g, 94%). Spectroscopic data were consistent with the literature.[56]

Synthesis of triphenyl(3-((tetrahydro-2*H*-pyran-2-yl)oxy)-propyl)phosphonium bromide (15). To a solution of 3-bromopropan-1-ol (5.01 g, 36 mmol) and 3,4-dihydro-2*H*-pyran (12.00 g, 142 mmol) in 140 mL of CH_2Cl_2 at 20 °C was added *p*-TsOH (0.05 g, 0.26 mmol) and the mixture was stirred at RT for 2 h. The solution was then diluted with 200 mL of Et_2O and washed successively with solutions of saturated $NaHCO_3$ (200 mL), water (200 mL) and brine (200 mL). The aqueous phase was worked-up with fresh Et_2O and the organic phases were combined and dried over Na_2SO_4. The mixture was

concentrated under reduced pressure and chromatographed on silica gel (*n*-hexane–AcOEt, 9 : 1) to afford the protected alcohol (6.01 g, 75.0%, TLC R_f = 0.26) as a colorless oil. GC-MS (EI^+): *m*/*z*(%): 41(79), 56(40), 85(100), 120(15), 221(50), 223(53) [M^+]. The alkyl bromide (3.04 g, 13.44 mmol) was dissolved in 15 mL of acetonitrile and triphenylphosphine (4.23 g, 16.13 mmol), and K_2CO_3 (1.01 g, 7.24 mmol) was added and the mixture was refluxed overnight (*ca*. 14 h). The precipitate was filtered off and the filtrate was poured into 150 mL of Et_2O to obtain the solid Wittig salt. After filtration the solid was washed with 100 mL of fresh Et_2O. The product (**15**) (5.81 g, 89%) was dried under vacuum and kept in a desiccator over $CaCl_2$ until use. ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 7.63–8.12 (m, 15H), 4.53 (br. s., 1H), 3.56–3.78 (m, 4H), 3.49 (dt, *J* = 9.9, 6.3 Hz, 1H), 3.43–3.80 (m, 6H), 1.65–1.87 (m, 3H), 1.54–1.64 (m, 1H), 1.34–1.52 ppm (m, 4H); ^{13}C NMR (DMSO-d_6, 100 MHz): δ = 135.9, 135.8, 134.2, 134.1, 130.9, 130.8, 119.0, 118.1, 100.5, 64.2, 30.9, 25.3, 20.0 ppm.

Synthesis of methyl (*Z*)-2-(3-oxo-2-(5-((tetrahydro-2*H*-pyran-2-yl)oxy)pent-2-en-1-yl)cyclopentyl)acetate (9). A 100 mL flask equipped with rubber septum was charged with (**15**) (5.23 g, 10.78 mmol, 1.1 equiv.) and flushed with dry argon. Potassium bis(trimethylsilyl)amide (KHMDS, 17 mL, 0.7 M in toluene, 11.86 mmol, 1.2 equiv.) and THF (35 mL) in that order were added to reach a final concentration of the ylide of approximately 0.2 M. The mixture was stirred at room temperature for 15 min and then cooled to −78 °C. The aldehyde (**8**) (1.94 g, 9.8 mmol, 1 eq.) in dry THF (15 mL) was added dropwise *via* a syringe and the reaction was stirred at −78 °C for an additional hour. The mixture was then filtered through a fritted funnel and *n*-hexane (100 mL) was added, the precipitate was filtered off, and the residual solids were combined and washed twice with *n*-hexane (50 mL portions). The filtrates were combined and concentrated under reduced pressure. Purification by flash chromatography (AcOEt–*n*-hexane, 1 : 2) provided (**9**) (2.21 g, 70%) as a colorless oil. ^{1}H NMR ($CDCl_3$, 500 MHz) mixture of diastereoisomers: δ = 5.44–5.55 (m, 1H), 5.36–5.44 (m, 1H), 4.58 (m, 1H), 3.85 (m, 1H), 3.64–3.77 (m, 1H), 3.69 (s, 3H), 3.46–3.53 (m, 1H), 3.40 (dtd, *J* = 9.5, 6.8(×2), 2.5 Hz, 1H), 2.64–2.74 (m, 1H), 2.18–2.43 (m, 7H), 2.11 (ddd, *J* = 18.7, 11, 9 Hz 1H), 1.87–1.95 (m, 1H), 1.76–1.86 (m, 1H), 1.63–1.74 (m, 1H), 1.44–1.61 ppm (m, 6H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 218.9, 172.6, 128.5, 127.8, 99.0, 67.0, 62.5, 54.1, 51.8, 38.9, 38.2, 37.8, 30.8, 28.2, 27.3, 25.9, 25.6, 19.8 ppm.

Synthesis of (2-(2-((*Z*)-5-hydroxypent-2-en-1-yl)-3-oxocyclopentyl)acetyl)-L-isoleucine, 12-OH-JA-Ile (3). Compound (**9**) (0.64 g, 1.73 mmol) was dissolved in 5 mL of MeOH, and 12 mL of NaOH (0.3 M) was then added. The reaction was heated at 70 °C for 1 h, cooled and diluted with 25 mL of water. A solution of HCl (1 M) was employed to adjust the pH to 3–4. The aqueous phase was then extracted three times with AcOEt (25 mL). The combined organic extracts were washed with brine and dried over anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure afforded (**10**) (0.45 g, 100%), which was used in the next step without any further purification.

Crude (**10**) (0.16 g, 0.50 mmol) and TEA (0.06 g, 0.54 mmol, 1.1 equiv.) were dissolved in THF (4 mL) and ethyl chloroformate (0.06 g, 0.55 mmol, 1.1 eq.) was added under stirring at 0 °C. After 5–10 min, L-Ile (0.13 g, 1.00 mmol, 2 eq.) dissolved in NaOH (4 mL, 0.3 M) was added and stirring was continued for 30 min at RT. The reaction mixture was then acidified with HCl (1 M) to pH around 3–4 and extracted with AcOEt. The combined organic extracts were dried over $MgSO_4$, and removal of solvents afforded (**11**) (0.18 g, 86%). Crude (**11**) (0.10 g, 0.23 mmol) and pyridinium *p*-toluenesulfonate (6 mg, 0.02 mmol) were dissolved in EtOH (2 mL) and stirred for 2 h at 55 °C. Next, 20 mL of water were added and the reaction was extracted three times with AcOEt (20 mL), and the combined organic extracts were washed with brine and dried over $MgSO_4$. After evaporation of the solvent and flash chromatography (AcOEt–2-propanol–AcOH, 32 : 2 : 1), compound **3** (70 mg, 90%) was obtained as a thick pale yellow oil. HRMS (ESI^--TOF): *m*/*z* = 338.1974 [M − H] (calc. for $C_{18}H_{28}NO_5$, 338.1968); ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 6.73 (d, *J* = 8.3 Hz, 1H), 6.49 (br. s., 1H), 5.44 (m, 2H), 4.57 (dd, *J* = 8.3, 5.0 Hz, 1H), 3.68 (m, 2H), 2.70 (m, 1H), 2.25–2.44 (m, 6H), 2.20 (m, 2H), 2.11 (m, 1H), 1.92 (m, 2H), 1.48 (m, 2H), 1.20 (m, 1H), 0.95 (d, 6 Hz, 3H), 0.92 ppm (t, 7.3 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 220.0, 174.9, 172.7, 128.9, 128.6, 62.1, 56.7, 54.2, 41.5, 38.9, 38.1, 37.6, 30.8, 27.4, 25.7, 25.3, 15.7, 11.7 ppm.

Synthesis of (4*S*,*Z*)-4-((*S*)-sec-butyl)-3,4,8,11,11a,13,14,14a-octahydro-1*H*-cyclopenta[*g*][1]-oxa[4]azacyclotridecine-2,5,12-(7*H*)-trione, JA-Ile-lactone (4). *Activation of the carboxyl group*: ethoxyacetylene (48 μL, 0.718 g mL^{-1} in hexanes, 0.49 mmol, 4 equiv.) and dichloro(*p*-cymene)ruthenium(II) dimer (1 mg, 0.002 mmol) were dissolved in dry acetone (3 mL) under an atmosphere of argon at 0 °C. 12-OH-JA-Ile (**3**) (42 mg, 0.12 mmol) dissolved in acetone (3 mL) was added slowly and the mixture was stirred for 1 h at RT. The reaction was then filtered through a short pad of silica and eluted with AcOEt. Evaporation of the solvent afforded the desired EVE derivative. *Macrolactonization*: *p*-TsOH (230 μL, 0.05 M in EtOH) was diluted in 1,2-dichloroethane (DCE, 20 mL), the solution was warmed to 50 °C and the previously obtained EVE (in 5 mL of DCE) was injected dropwise for 2 h. The mixture was stirred for another 6 h and worked up. Flash chromatography (AcOEt–*n*-hexane, 7 : 3) afforded JA-Ile-lactone (**4a**) (11.6 mg) and JA-Ile-lactone (**4b**) (13.7 mg). The total yield was 64% (25.3 mg). Recrystallization was carried out as described above in the results and discussion section.

JA-Ile-lactone (4a). Silica gel TLC R_f = 0.26; m.p. (from AcOEt–*n*-hexane, uncorrected) 188–189 °C; HRMS (ESI^+-TOF): *m*/*z* = 344.1846 $[M + Na]^+$ (calc. for $C_{18}H_{27}NNaO_4$, 344.1832); ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.71 (d, *J* = 8.8 Hz, 1H), 5.43 (m, 1H), 5.18 (m, 1H), 4.57 (ddd, *J* = 10.8, 4.3, 3.4 Hz, 1H), 4.49 (dd, *J* = 8.9, 6.7 Hz, 1H), 3.90 (td, *J* = 11.0, 1.7 Hz, 1H), 2.63 (dd, *J* = 12.1, 2.2 Hz, 1H), 2.58–2.38 (m, 4H), 2.36–2.04 (m, 6H), 1.91 (m, 1H), 1.66 (m, 1H), 1.47 (ddq, *J* = 13.4, 7.5(×4) Hz, 1H), 1.16 (m, 1H), 0.94 (d, *J* = 7.0 Hz, 3H), 0.92 ppm (t, *J* = 7.3 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 220.0, 170.9, 170.6, 128.9,

128.4, 63.3, 57.3, 53.4, 43.0, 38.3, 37.6, 36.5, 28.6, 27.8, 25.7, 25.1, 15.7, 11.3 ppm.

JA-Ile-lactone (4b). Silica gel TLC R_f = 0.19; m.p. (from EtOH–acetone, uncorrected) 186–187 °C; HRMS (ESI$^+$-TOF): m/z = 344.1838 $[M + Na]^+$ (calc. for $C_{18}H_{27}NNaO_4$, 344.1832); ^{1}H NMR ($CDCl_3$, 500 MHz): δ = 5.62 (d, J = 8.1 Hz, 1H), 5.45 (m, 2H), 4.41 (m, 2H), 3.95 (ddd, J = 10.8, 6.4, 2.8 Hz, 1H), 2.60 (m, 2H), 2.48 (m, 1H), 2.37 (m, 3H), 2.25 (m, 3H), 2.06 (dd, J = 14.2, 10.3 Hz, 1H), 1.96 (m, 2H), 1.60 (m, 1H), 1.46 (m, 1H), 1.19 (m, 1H), 0.96 (d, J = 6.7 Hz, 3H), 0.93 ppm (t, J = 7.4 Hz, 3H); ^{13}C NMR ($CDCl_3$, 125 MHz): δ = 219.6, 171.4, 171.0, 129.3, 127.8, 64.2, 57.6, 55.9, 42.7, 37.8, 37.2, 36.1, 28.1, 27.1, 25.2, 25.2, 16.0, 11.5 ppm.

Crystal structure determination

The intensity data for the compounds were collected on a Nonius KappaCCD diffractometer using graphite-monochromated Mo-K_α radiation. Data were corrected for Lorentz and polarization effects but not for absorption effects.[57,58] The structures were solved by direct methods (SHELXS[59]) and refined by full-matrix least-squares techniques against F_o^2 (SHELXL-97).[59] All hydrogen atoms were located by difference Fourier synthesis and refined isotropically. All non-hydrogen atoms were refined anisotropically. Mercury 3.5.1 (Cambridge Crystallographic Data Centre, Build RC5) software was used for structure representations.

Crystal data for JA-Ile-lactone (4a). $C_{18}H_{27}NO_4$, M = 321.41 g mol^{-1}, colourless prism, size 0.075 × 0.054 × 0.048 mm^3, orthorhombic, space group $P2_12_12$, a = 15.6931(3), b = 21.8725(4), c = 5.0383(1) Å, V = 1729.38(6) Å^3, T = −140 °C, Z = 4, ρ_{calcd} = 1.234 g cm^{-3}, μ (Mo-K_α) = 0.86 cm^{-1}, F(000) = 696, 12 151 reflections in h(−20/20), k(−28/27), l(−6/6), measured in the range 2.27° ≤ Θ ≤ 27.48°, completeness Θ_{max} = 99.8%, 3985 independent reflections, R_{int} = 0.0599, 3378 reflections with $F_o > 4\sigma(F_o)$, 316 parameters, 0 restraints, R^1_{obs} = 0.0466, wR^2_{obs} = 0.0881, R^1_{all} = 0.0617, wR^2_{all} = 0.0946, GOOF = 1.164, Flack-parameter −1.4(8), largest difference peak and hole: 0.207/−0.192 e Å^{-3}.

Crystal data for JA-Ile-lactone (4b). $C_{18}H_{27}NO_4$, M = 321.41 g mol^{-1}, colourless prism, size 0.06 × 0.06 × 0.04 mm^3, orthorhombic, space group $P2_12_12_1$, a = 5.7917(3), b = 15.4852(7), c = 18.6976(9) Å, V = 1676.91(14) Å^3, T = −140 °C, Z = 4, ρ_{calcd} = 1.273 g cm^{-3}, μ (Mo-K_α) = 0.89 cm^{-1}, F(000) = 696, 27 196 reflections in h(−5/7), k(−18/19), l(−23/24), measured in the range 2.85° ≤ Θ ≤ 27.52°, completeness Θ_{max} = 99.7%, 3401 independent reflections, R_{int} = 0.0326, 3299 reflections with $F_o > 4\sigma(F_o)$, 316 parameters, 0 restraints, R^1_{obs} = 0.0296, wR^2_{obs} = 0.0749, R^1_{all} = 0.0309, wR^2_{all} = 0.0759, GOOF = 1.031, Flack-parameter −0.1(2), largest difference peak and hole: 0.219/−0.190 e Å^{-3}.

Supporting information available. Crystallographic data (excluding structure factors) has been deposited with the Cambridge Crystallographic Data Centre as supplementary publication CCDC 1004515 for JA-Ile-lactone (**4a**) and CCDC 1004516 for JA-Ile-lactone (**4b**).

Plant material and planting conditions

In the present study, we used wild-type *N. attenuata* Torr. Ex. Watson plants of the 31st inbred generation derived from seeds collected at the Desert Inn Ranch in Utah, UT, USA in 1988. Before planting, the seeds were surface sterilized and germinated on Gamborg's B5 media as described by Krügel *et al.*[60] Ten-day-old seedlings were transferred to Teku pots for another ten days (Pöppelmann GmbH & Co. KG, Lohne, Germany) before planting them into 1 L pots filled with washed sand. Twenty days later, 0.8 μmol of each compound (per plant) dissolved in lanoline paste was applied to the petioles of rosette-stage plants. The treatments were repeated every other day for five days to obtain nine treated leaves in total. Lanolin-treated plants were used as a negative control (n = 6).[61] The leaves were harvested 24 h after the last treatment, flash frozen in liquid nitrogen and stored at −80 °C until analyzed. Nicotine was quantified as previously described.[62,63] Plants were grown at 45–55% relative humidity and 24–26 °C during days and 23–25 °C during nights under 16 h of light. Plants were watered twice every day by an automatic irrigation system.

Statistics

The statistical tests were carried out with Sigma Plot 12.0 (Systat Software Inc., San Jose, CA, USA) using analysis of variance. Levene's and Shapiro–Wilk's tests were applied to determine error variance and normality. The Holm–Sidak *post hoc* test was used for multiple comparisons. To fulfill the assumptions for ANOVA, the data set was root square-transformed prior to analysis.

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Electronic Supplementary Material (ESI) for Organic & Biomolecular Chemistry.

Synthesis, structural characterization and biological activity of two diastereomeric JA-Ile macrolactones

Supporting Information

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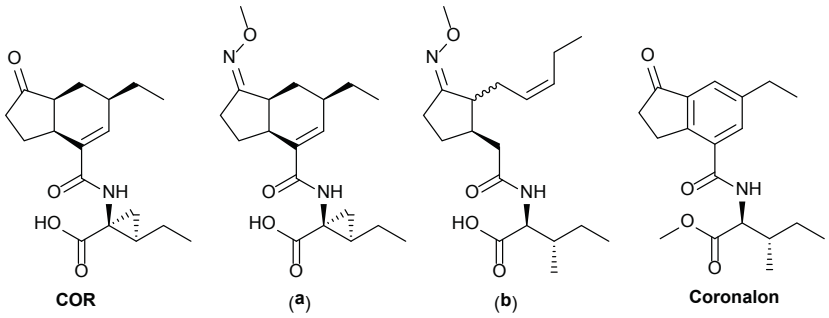
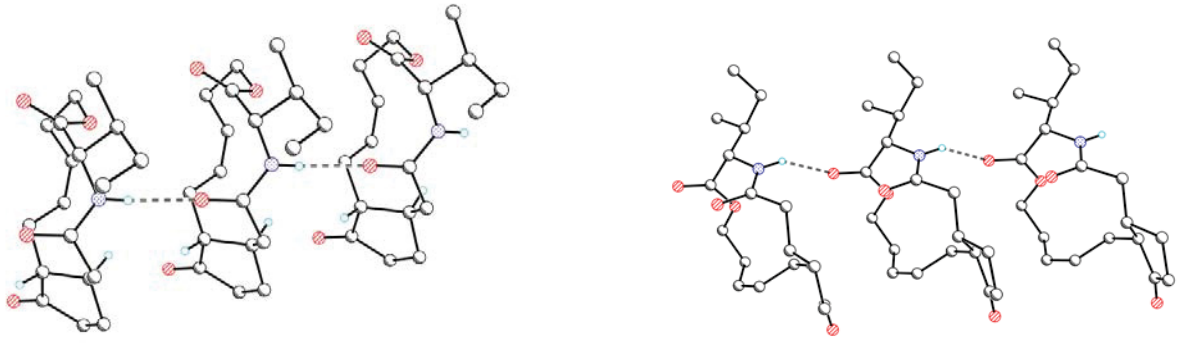


Fig. S1 Chemical structure of coronatine (**COR**), coronatine methyloxime (**a**), JA-Ile-methyloxime (**b**) and coronalon.

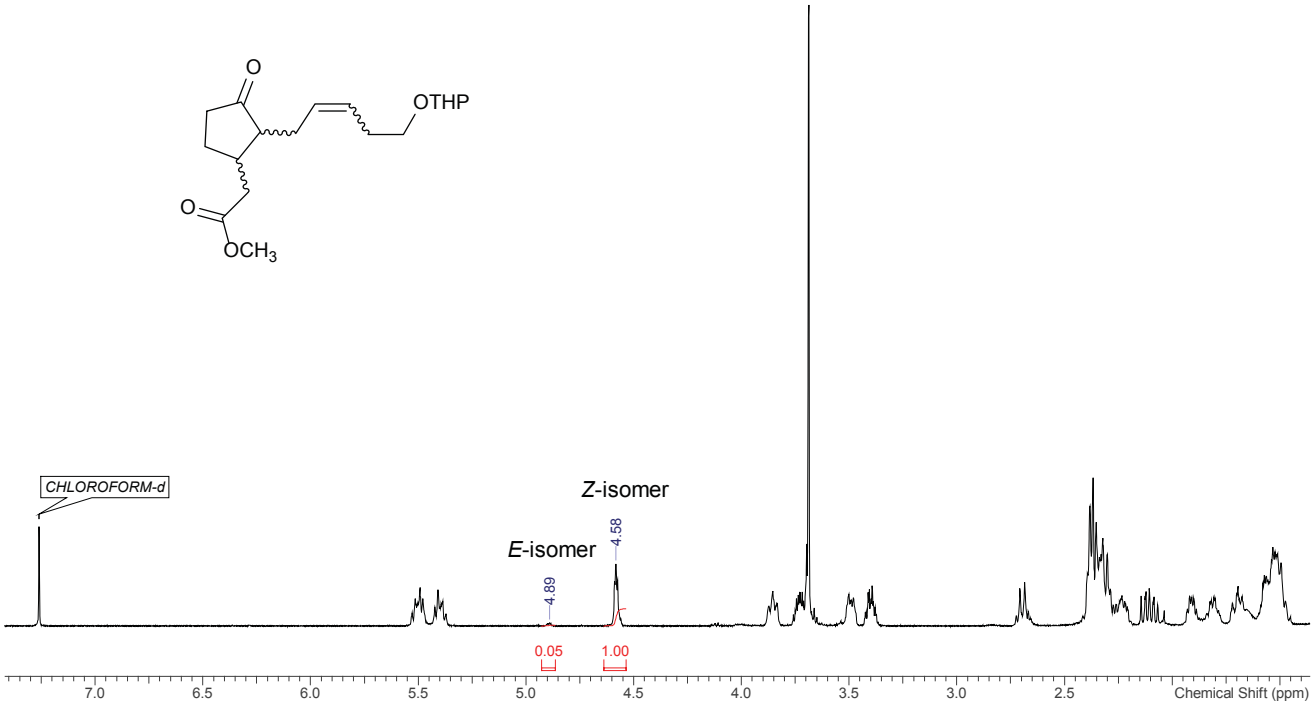


A) B)

Figure S2. Both lactones show a chain-like packing. A) JA-Ile-lactone (**4a**). B) JA-Ile-lactone (**4b**)

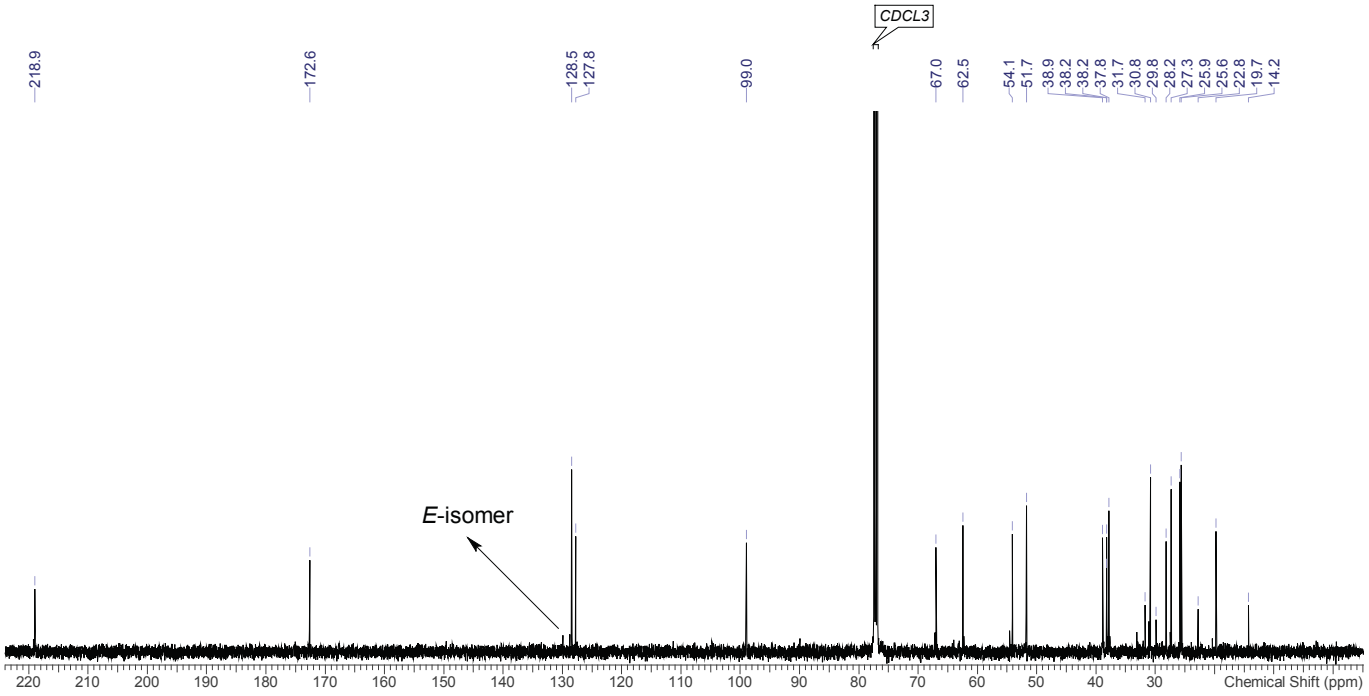
COPY OF NMR SPECTRA OF IMPORTANT COMPOUNDS

1 Compound (**9**)



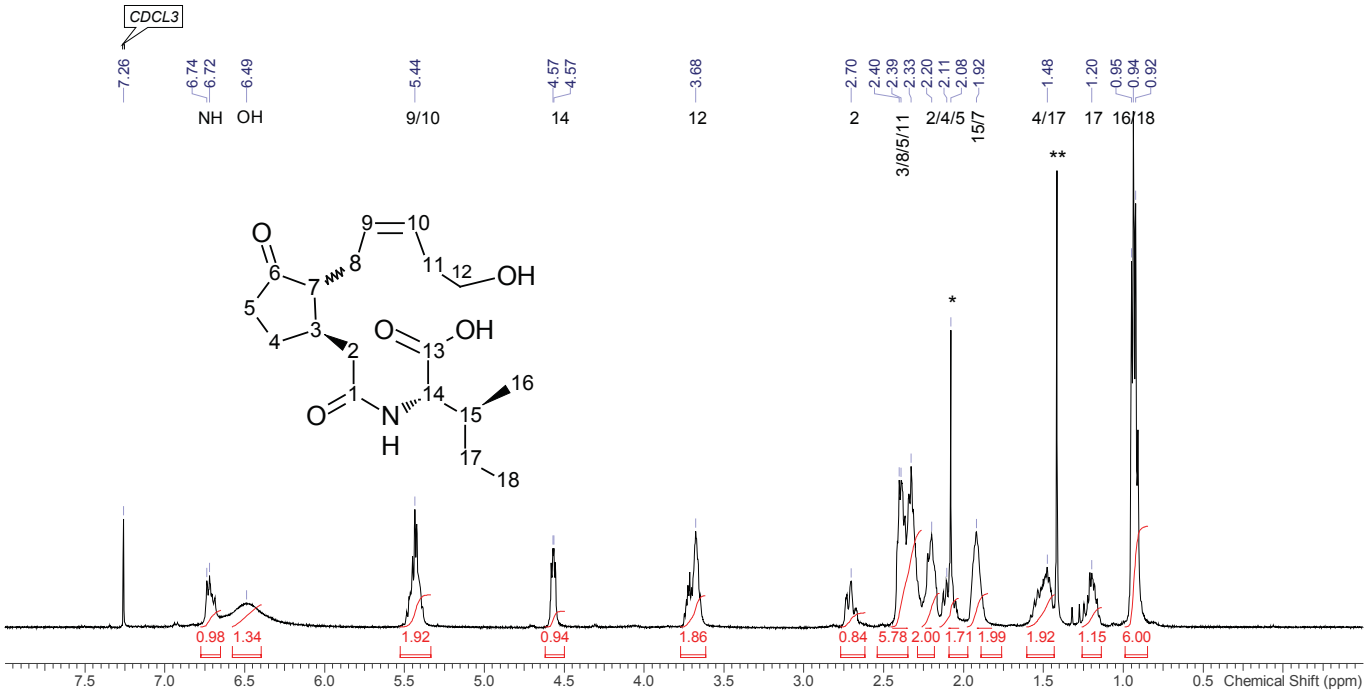
2

3 ^{1}H NMR in $CDCl_3$.



^{13}C NMR in $CDCl_3$.

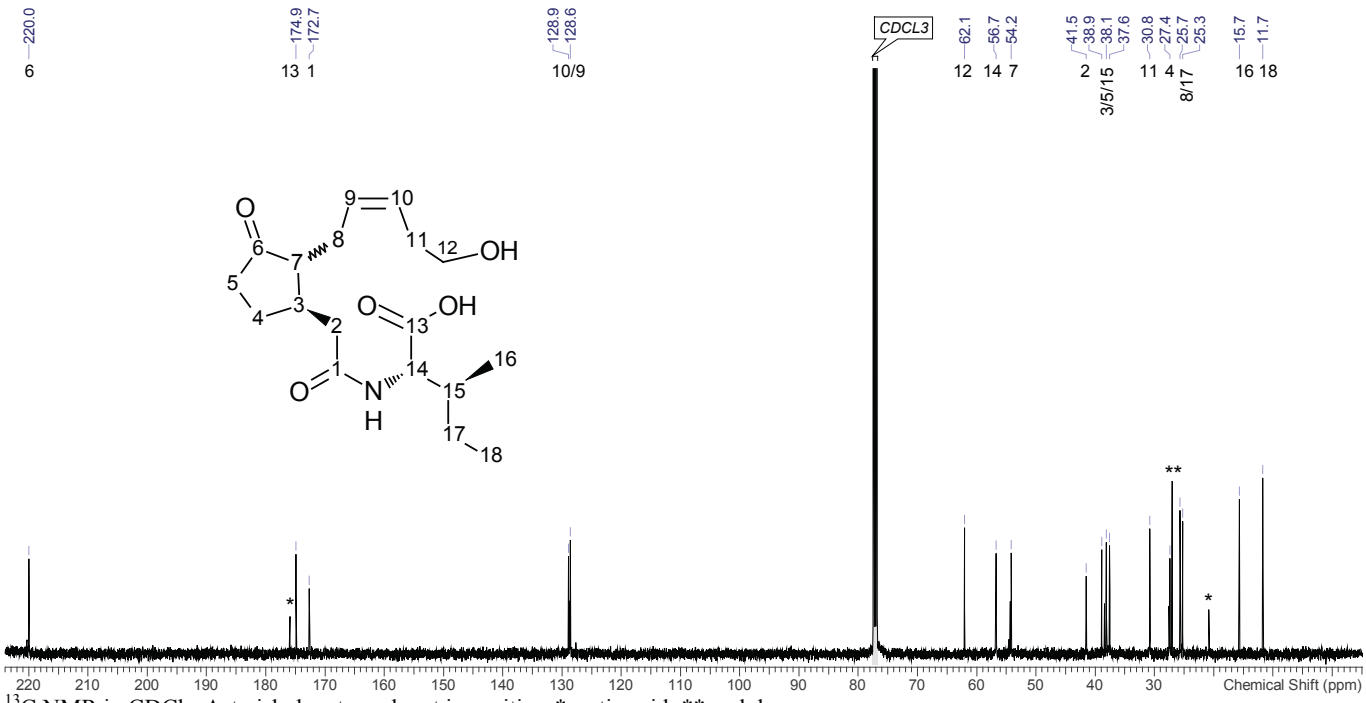
1 12-OH-JA-Ile (**3**)



2

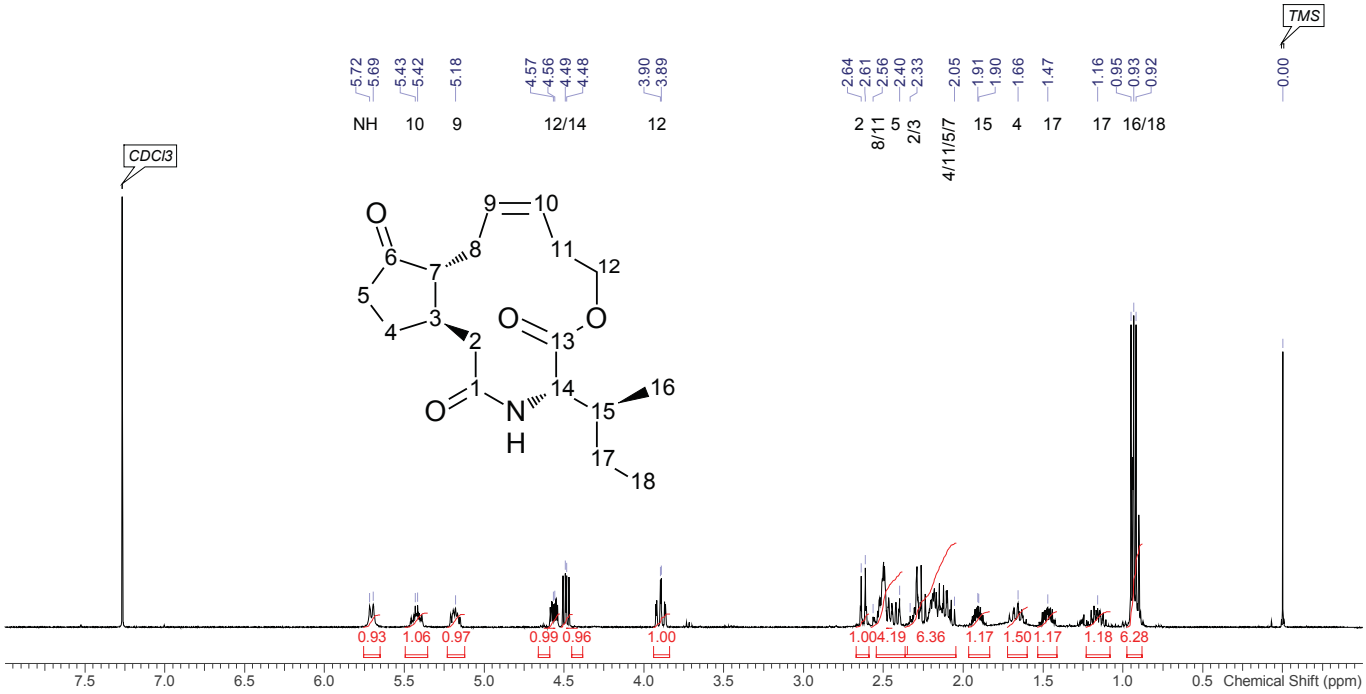
3 ^{1}H NMR in $CDCl_3$. Asterisk denotes solvent impurities. *AcOH; **cyclohexane

4

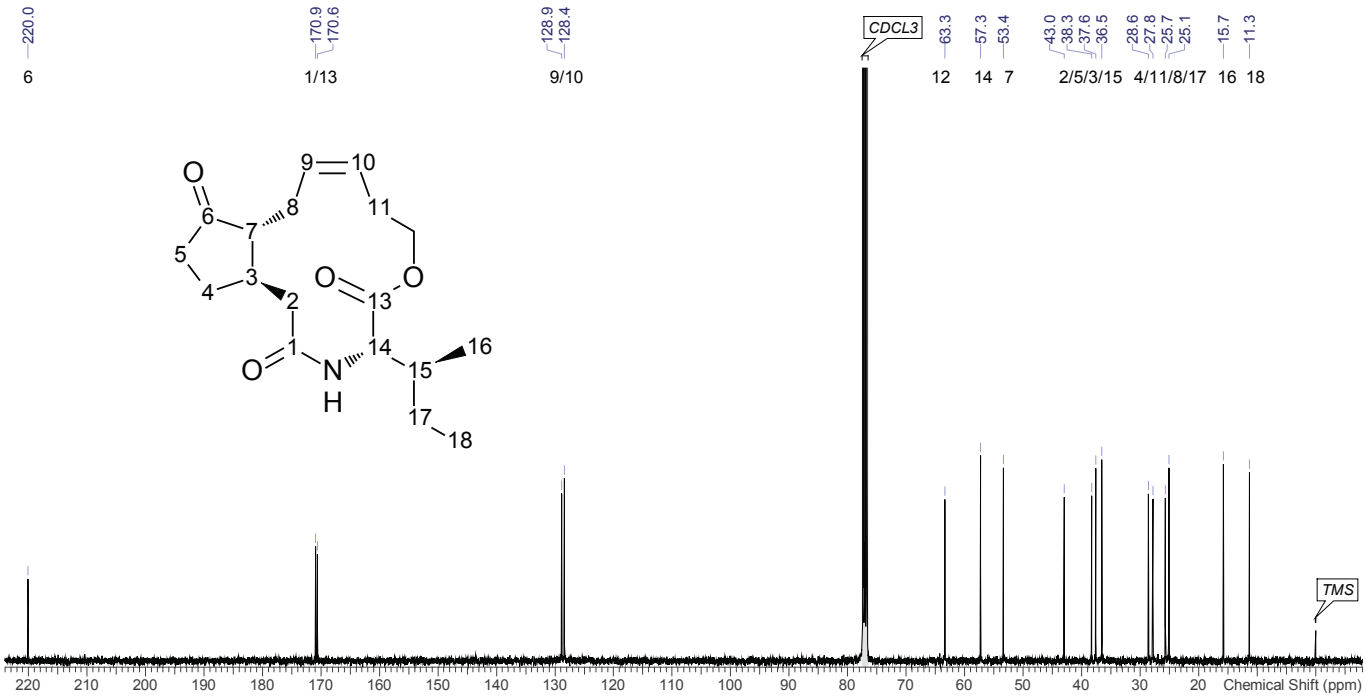


^{13}C NMR in $CDCl_3$. Asterisk denotes solvent impurities. *acetic acid; **cyclohexane

JA-Ile-Lactone (**4a**)

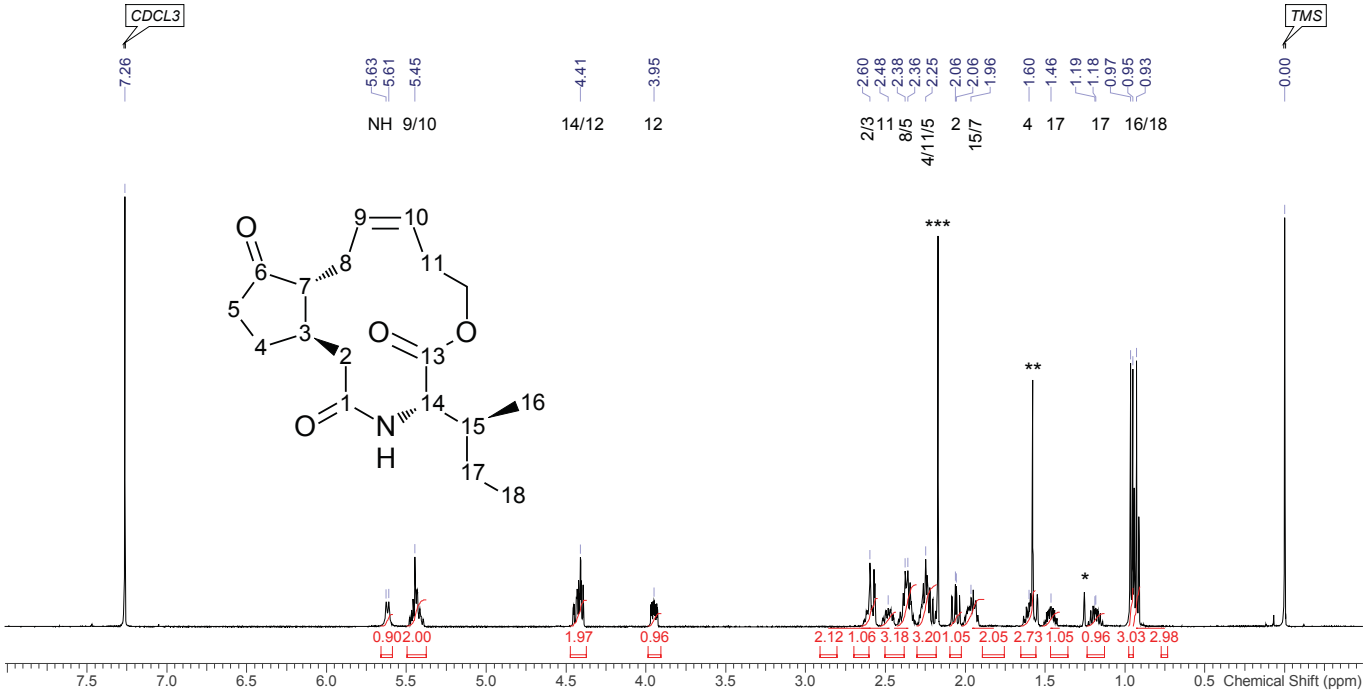


^{1}H NMR in $CDCl_3$.



^{13}C NMR in $CDCl_3$.

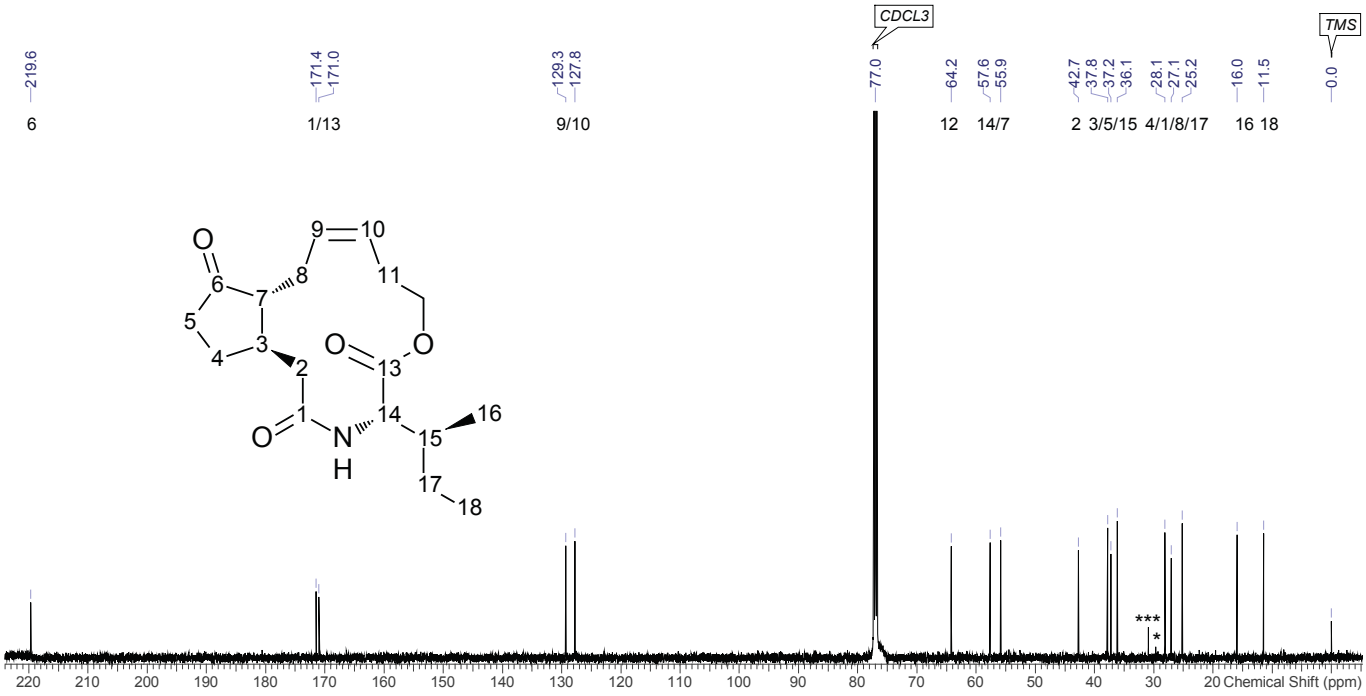
1 JA-Ile-Lactone (**4b**)



2

3 ^{1}H NMR in $CDCl_3$. Asterisk denotes minor impurities. *H grase; ** Water; ***Acetone

4



^{13}C NMR in $CDCl_3$. Asterisk denotes minor impurities. *H grase; ***Acetone.

7. Unpublished results

Inactivation of JA-Ile after ω-hydroxylation: Steric hindrance may be the key

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(…in preparation)

Abstract

N-jasmonoyl-L-isoleucine (JA-Ile, **2**) is a plant hormone that regulates important processes in higher plants such as growth and development. On the one hand, JA-Ile activates plant defenses that protect plants against herbivorous insects. On the other hand, activation of plant defenses by JA-Ile compromises plant protection against hemibiotrophic plant pathogens such as *P. syringae*. The JA-Ile (**2**) receptor (which crystal structure is known) is a ternary complex containing the F-box protein COI1 (coranatine insensitive 1) and a member of the JAZ (jasmonate ZIM domain) family of repressors. JA-Ile (**2**) is inactivated upon ω-hydroxylation by JA-Ile-12-hydroxylases forming 12-OH-JA-Ile (**3**). The mechanistic details of the JA-Ile inactivation processes remain yet unclear. Recently, we reported that a macrolactone (JA-Ile-lactone, **7**) prepared from 12-OH-JA-Ile (**3**) is biologically active. This observation prompted us to investigate the JA-Ile (**2**) inactivation mechanism upon ω-hydroxylation in *Arabidopsis thaliana*. Here, we designed JA-Ile derivatives in order to further dissect the JA-Ile signaling pathway. Our results suggest that (i) impaired activity of 12-OH-JA-Ile (**3**) may be due to steric hindrance caused by the hydroxyl group, (ii) the JA-Ile-lactone (**7**) is capable of activating a subset of jasmonate responsive genes (JRGs) similarly to JA-Ile (**2**), and (iii) the free carboxyl group of JA-Ile (**2**) is not essential to activate JRG expression. Results of this study provide evidence for the great potential of

rationally designed JA-Ile derivatives to study the JA-Ile (**2**) perception and signaling mechanism. Molecular modelling studies which will provide further insights into the JA-Ile-lactone perception mechanism and physiology are in progress.

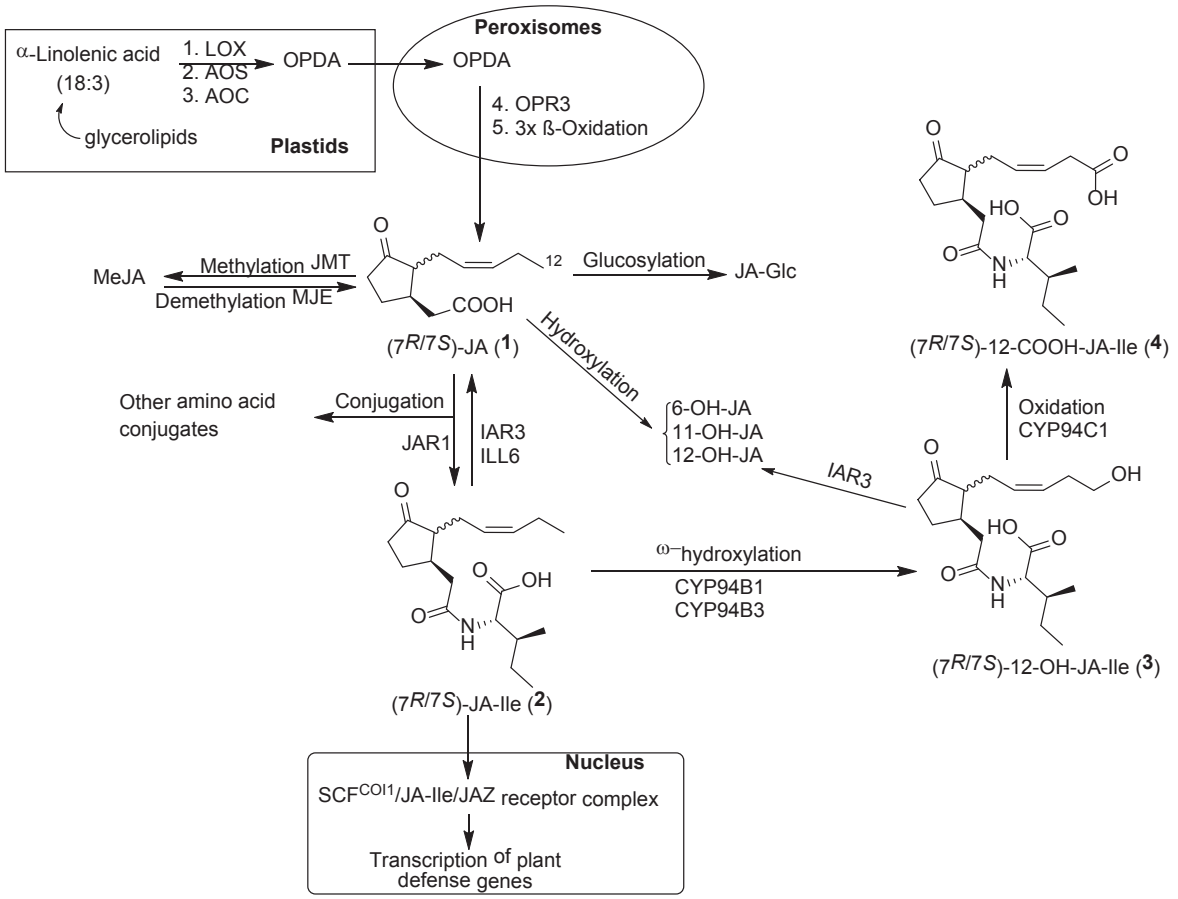
Introduction (see also chapter 1)

While efforts have been directed toward understanding the biology of the JA-Ile inactivation upon ω-hydroxylation, the apparently simple chemistry (single hydroxylation at C12 of JA-Ile) responsible for the change in JA-Ile bioactivity remains unexplored. In our search for new bioactive jasmonates that can enhance plant protection against herbivory, a macrolactone (obtained from inactive 12-OH-JA-Ile) which showed strong biological activity was prepared [86]. The surprisingly high bioactivity of the JA-Ile-lactone (**7**), in addition to the possibility of manipulating the JA-Ile signaling pathway by chemically modified ligands [87], prompted us to investigate the inactivation mechanism of JA-Ile upon hydroxylation at C12 (Scheme 1). In the present study, a rational-designed synthetic approach combined with RT-PCR and molecular modeling (in progress) was employed to investigate the physicochemical principles governing the inactivation mechanism of JA-Ile (**2**) *via* the ω-hydroxylation pathway in *A. thaliana*.

Results

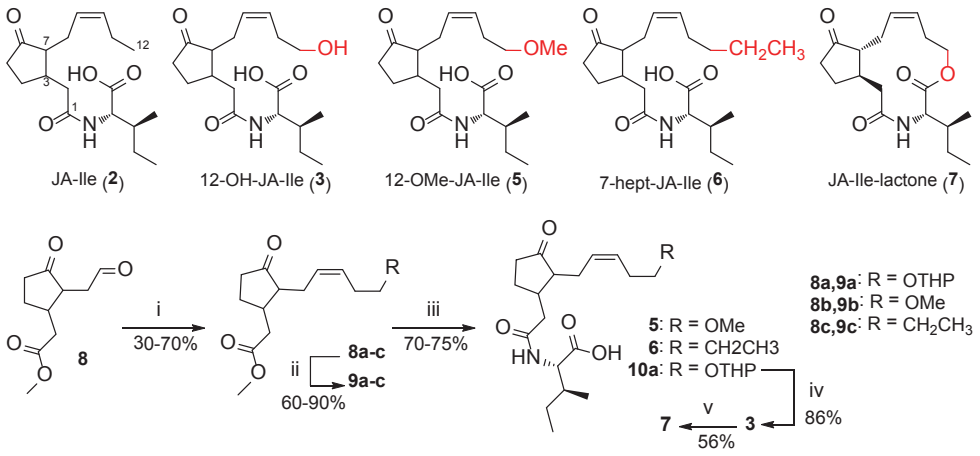
Rational design and synthesis of the JA-Ile (2) derivatives

The impaired bioactivity of 12-OH-JA-Ile (**3**) can be explained by at least three reasons; i) different conformation of the hydroxylated molecule compared to JA-Ile (**2**) (e.g. intramolecular hydrogen bond of the ─OH group of **3** with one of the carbonyl groups in the same molecule) that leads to weaker or no interactions of 12-OH-JA-Ile (**3**) with the receptor complex, ii) different interaction mechanism of 12-OH-JA-Ile (**3**) with the receptor (e.g. possible electrostatic interactions of the ─OH at C12 with amino acid residues of COI1), and iii) steric hindrance due to the replacement of a hydrogen atom by the bulkier hydroxyl group. To explore these hypotheses we have synthesized appropriate JA-Ile (**2**) derivatives (Scheme 2) and tested their capability of inducing JRG expression.



Scheme 1. Simplified scheme of the biosynthetic, signalling and catabolic pathways of jasmonates. JA biosynthesis starts with the release of LA acid from membrane lipids by lipases in the plastid. The succession of LOX, AOS, and AOC catalyzes the formation of *cis*-OPDA. c*is*-OPDA is transported into the peroxisomes where after its reduction by OPR3 and three rounds of β-oxidation is converted into JA (**1**). In the cytosol, JAR1 catalyzes the conjugation of L-Ile to JA (**1**) producing JA-Ile (**2**). The model proposes the formation of the SCF^{COI1}/JA-Ile/JAZ receptor complex in the nucleus resulting in jasmonate perception and activation of gene expression. Turnover of JA-Ile (**2**) is carried out by amidohydrolases and members of the CYP94 family. The compounds are shown in the (7*S*) and (7*R*) stereochemistry occurring *in planta* as indicated by the wavy bonds.

Compounds **5**-**7** are designed to explore the influence of different modifications at the terminus of the pentenyl side chain of JA-Ile (**2**). A procedure for the synthesis of JA-Ile-lactone (**7**), which also allows the preparation of the 12-modified JA-Ile derivatives **3**, **5**, and **6**, was reported by our group recently [86]. In compound **5** (12-OMe-JA-Ile) the hydroxyl group of **3** was replaced by a methoxy group; thereby, the capability of hydrogen bonding of the –OH (as a proton donor) was blocked. If a hydrogen bond involving the C12 hydroxyl group of **3** is responsible for the reduced bioactivity of this molecule, in compound **5** this effect



Scheme 2. Compounds employed in this study. Synthetic route to the compounds. Reagents and conditions: (i) **12**, **13** or **14** (~ 0.2 M), KHMDS (1.2 equiv.), THF, -78 °C, then **8**, -78 °C to RT; (ii) NaOH (~ 0.3 M), MeOH, 70 °C; (iii) Ethyl chloroformate ($ClCOOC_2H_5$, 1.1 equiv.), TEA, THF, L-Ile (2 equiv.), 0 °C; (iv) PPTS (10 mol%), EtOH, 55 °C; (v) Ethoxyacetylene (4 equiv.), [Ru(*p*-cymene)Cl_2]$_2$, Acetone, 0 °C, then *p*TsOH (0.05 M) in 1,2-dichloroethane, high dilution, 50 °C.

should be absent, and **5** should induce JRG expression similar to JA-Ile (**2**).The derivative 7-hept-JA-Ile (**6**) has no heteroatom on C12; thus, no strong electrostatic interactions are possible at the terminus of the side chain. Besides, the heptenyl side chain of **6** may be accommodated by hydrophobic interactions in the binding pocked of the receptor similarly to the pentenyl side chain of JA-Ile (**2**) [67]. Therefore, the hypothesis is that compound 7-hept-JA-Ile (**6**) induces JRG expression, unless steric hindrance by the larger heptenyl compared to a pentenyl side chain causes inactivity of the molecule. JA-Ile-lactone (**7**) has a rigid conformation as it was previously determined by x-ray [86]. The conformational change and the steric hindrance hypotheses can be explored at once with this compound. If the —OH group of 12-OH-JA-Ile (**3**) can form an intramolecular hydrogen bond to the carboxyl group of the L-Ile moiety, the conformation of **3** could be similar to that of JA-Ile-lactone (**7**). In this case, both functional groups (—OH and —COOH) are near in space due to the H-bond, while in the lactone **7** they are even closer to each other because of the covalent ester bond. Although the pentenyl side chain of the lactone **7** contains an oxygen atom on C12, the side chain is neither extended nor flexible like in 12-OH-JA-Ile (**3**) or 12-OMe-JA-Ile (**5**). Therefore, a possible steric hindrance of the substituent at C12 should be less for **7** due to its more compact structure, which may fit into the receptors binding pocket.

Synthetic compounds where obtained in moderate to good yields (Scheme 2) following the procedure previously described in [86]. All compounds were employed as a mixture of diastereomers in bioassays with the exception of lactone **7**, which was diastereomerically pure.

Expression levels of JRGs after plant treatments with compounds 2, 3, 5, 6, and 7

First, a subset of marker JRGs that are significantly upregulated 16 h after spraying *A. thaliana* WT plants with a solution of JA-Ile (**2**) was selected (Figure S1, SI). After plant treatments with compounds **2** (active), **3** (inactive), **5**, **6** and **7** the expression levels of the selected marker JRGs ─ *VSP2*, *MYC2*, *THI2.1*, *AOS* ─ were measured and compared.

It was shown that the 12-OMe-JA-Ile (**5**) derivative, which cannot donate a proton to form a hydrogen bond, induces gene expression weakly, similarly to partially inactive 12-OH-JA-Ile (**3**) (Figure 1). This suggests that partial inactivation of JA-Ile (**2**) upon hydroxylation at C12 is not caused by conformational changes resulting from an intra- or intermolecular hydrogen bond where the ─OH group of **3** donates a proton. Interestingly, compound 7-hept-JA-Ile (**6**) was also inactive in the activation of JRGs expression, which suggests that a heptenyl side chain cannot be accommodated into the active site of the receptor even though its polarity is similar polarity of the pentenyl side chain of JA-Ile (**2**). This outcome lead to two important observations i) the electrostatic loading of the pentenyl side chain terminus is not crucial for inactivation of JA-Ile (**2**), and ii) the length of the side chain on C3 of JA-Ile (**2**) is of great importance for bioactivity, which is in agreement with previous studies on JA (**1**) derivatives [24,25].

JA-Ile-lactone (**7**) can induce nicotine accumulation in a NaCOI1 dependent manner in *N. attenuata* (Fig. S2, SI). Therefore, the hypothesis is that this compound can also induce the expression of COI1-dependent JRG [66,88] in *A. thaliana*. As it was expected, the lactone **7** was as active as JA-Ile (**2**) inducing the expression of the selected marker genes (Figure 2). A compound like **7**, with a more compact structure than 12-OH-JA-Ile (**3**), seems to be able to promote COI1-JAZ interactions and activates JRGs expression. All gene expression data

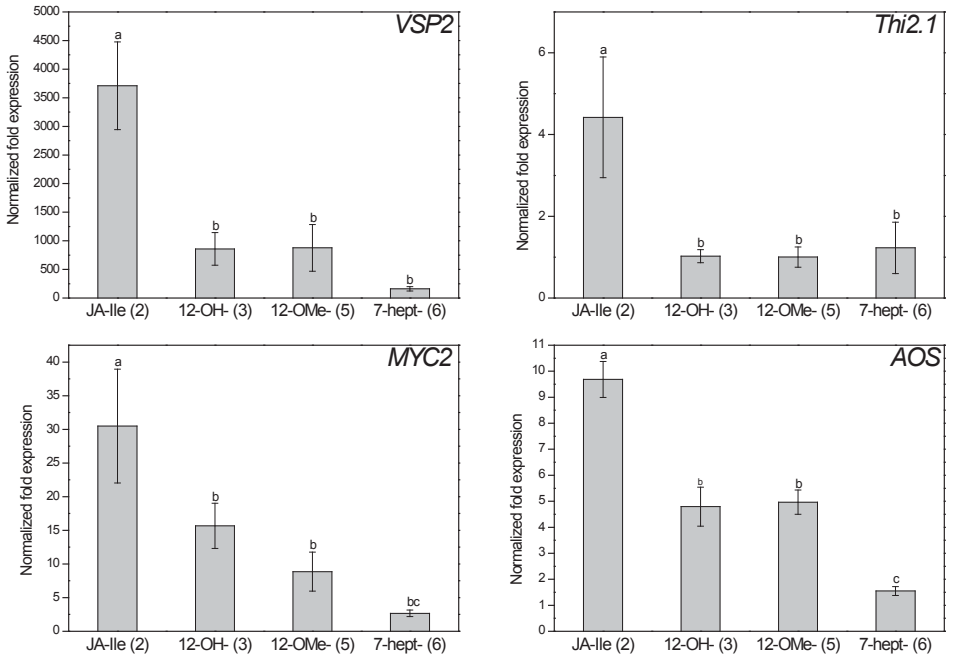


Figure 1. Replacement of a hydrogen at C12 in JA-Ile (2) negatively affects the gene expression capability of the compound. Transcript levels of the marker JRGs 16 h after spraying plant leaves with JA-Ile (**2**), 12-OH-JA-Ile (**3**), 12-OMe-JA-Ile (**5**) or 7-hept-JA-Ile (**6**). Ethanol 0.4% was sprayed on control plants. Transcript abundance was determined by real-time PCR analysis and normalized to the *RPS18B gene*. Fold change of respective genes was calculated relative to the ethanol control. Different letters indicate significant statistical differences among treatments ($P < 0.05$, SNK test).

obtained with these compounds (**2**-**7**) suggest that the inactivation of JA-Ile (**2**) upon the ω-hydroxylation pathway is likely to be caused by steric hindrance after the introduction of the —OH group at C12.

Molecular modeling (docking) studies with the different ligands

Molecular modeling studies aimed to achieve a better understanding of the multicomponent receptor complex of JA-Ile (**2**) are currently in progress. Even though the binding-pocket of COI1 is difficult to model, the structure of the lactone **7** is more rigid than that of JA-Ile (**2**). With a rigid molecule like **7** as the ligand, the COI1 receptor should adapt its conformation to accommodate the molecule in the binding pocket. Therefore, the modeling of the tripartite system COI1-lactone**7**-JAZ is simpler than the system containing JA-Ile (**2**) as the ligand. The crystal structure of COI1 is known [67] and the crystal structure of the JA-Ile-lactone (**7**) was

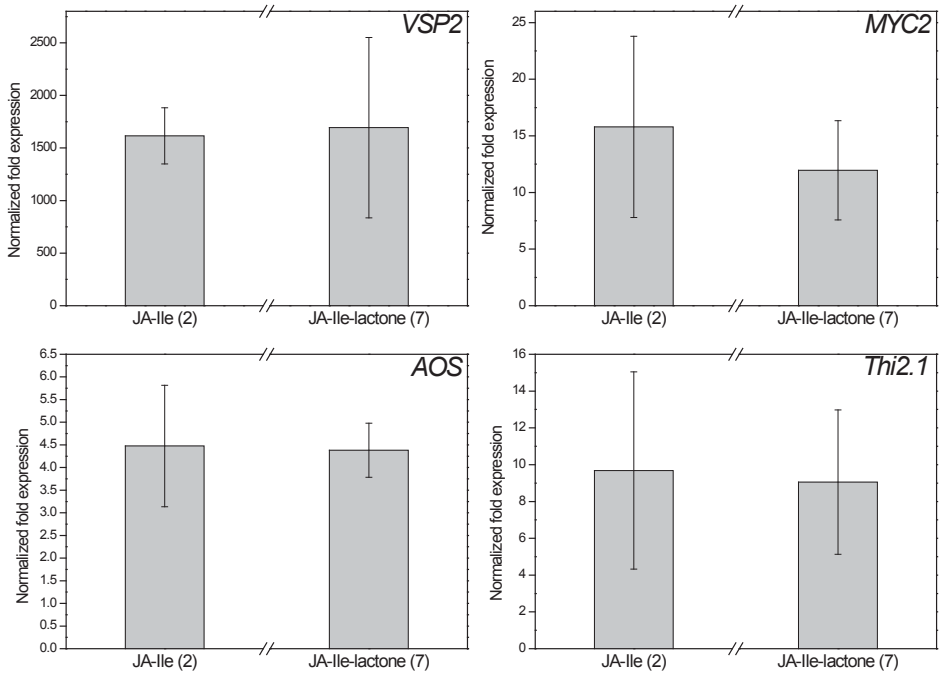


Figure 2. JA-Ile-lactone (7) activates gene expression similarly to the bioactive jasmonate JA-Ile (2). Transcript levels of the marker JRGs 16 h after spraying plant leaves with JA-Ile (**2**), or JA-Ile-lactone (**7**). Ethanol 0.4% was sprayed on control plant leaves. Transcript abundance was determined by real-time PCR analysis and normalized to the *RPS18B gene*. Fold change of respective genes was calculated relative to the ethanol control. Different letters indicate significant statistical differences among treatments ($P < 0.05$, SNK test).

elucidated recently [86]. The results of the docking calculation will be added to the work in due course.

Discussion

The synthetic JA-Ile-lactone (**7**) obtained from the inactive jasmonate 12-OH-JA-Ile (**3**) activates the expression of the JRGs *VSP2*, *MYC2*, *THI2.1*, and *AOS* in a similar way as JA-Ile (**2**) does. JA-Ile-methyl-ester and coronalon (Fig. S1, supplementary information for article III) are analogues of JA-Ile (**2**) which exhibit strong and diverse biological activities [39,78,89]. Currently, it is accepted that the ester bond of these compounds and other bioactive indanoyl derivatives is cleaved *in vivo* generating the free carboxyl group as it was shown for MeJA [90]. As JA-Ile-lactone (**7**) and JA-Ile (**2**) showed the same profile in our gene expression assays, it must be pointed out that an *in vivo* cleavage of the ester bond of the

lactone **7** leads to the inactive 12-OH-JA-Ile (**3**). This strongly suggests that a free carboxyl group is not crucial for JA-Ile (**2**) or its analogues to interact with COI1 and activate JRG expression.

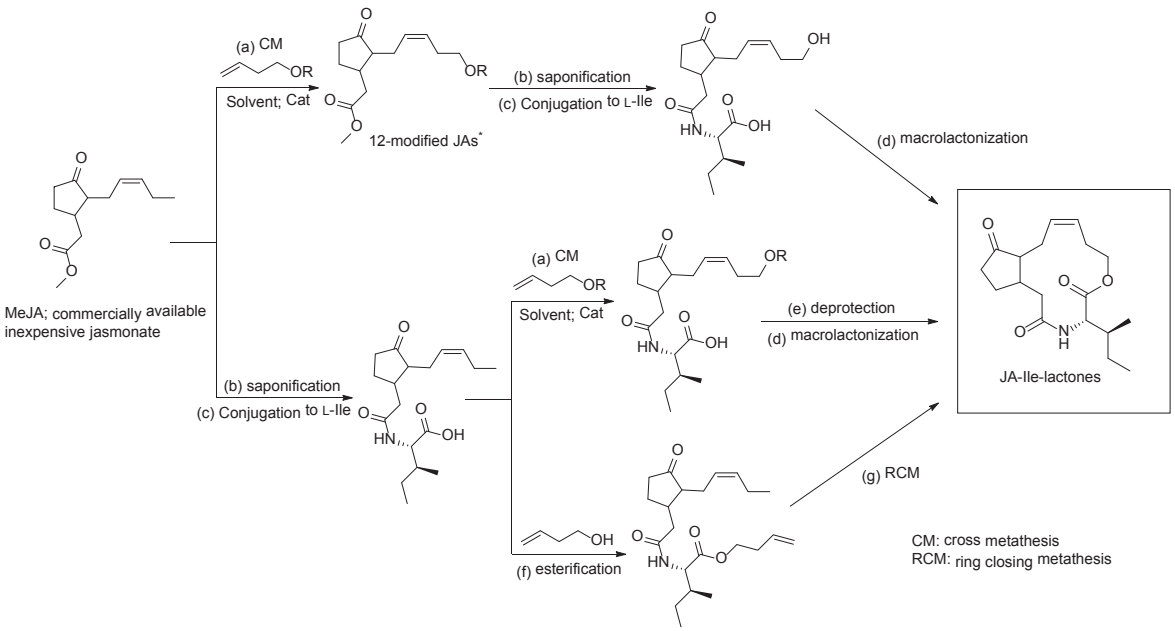
The results of gene expression assays suggest that the inactivation of the bioactive jasmonate JA-Ile (**2**) upon ω-hydroxylation is more likely to be caused by the steric hindrance of the —OH group introduced at C12. The pentenyl side chain of JA-Ile (**2**) is precisely accommodated in the receptors binding pocket [67]. The enlargement of the pentenyl side chain (e.g. by introduction of a hydroxyl group at C12) seems to interfere with the recognition of the molecule by the SCF^{COI1} receptor complex.

Macrolactones are compounds widely spread among natural products, e.g. macrolides [91]. Jasmine ketolactone [92] is a macrolactone occurring in nature and it is an analogue to JA-Ile-lactone (**7**) [86,93]. The fate of 12-OH-JA-Ile (**3**) — a potential precursor of the lactone **7** — in the cell is not fully understood. Therefore, it is tempting to speculate that a macrolactone like **7** might exist in nature.

The above mentioned results highlight the potential of synthetic organic chemistry tools to explore biological systems, in particular the jasmonates perception and signaling mechanisms. Moreover, outcomes provide further evidence that there is a possibility to manipulate the JA-Ile (**2**) signaling pathway by using rational designed jasmonates.

Future perspectives

The development of ruthenium-based catalysts, active in alkene metathesis, has enabled the efficient preparation of several natural products [94-96]. Jazmine ketolactone (Fig. 1, article III) has been efficiently prepared *via* alkene metathesis employing a ruthenium catalyst [97]. Furthermore, cross metathesis (CM) and ring-closing metathesis (RCM) can produce high yield of the targeted *Z*-isomer by using *Z*-selective Grubbs type of catalysts [98-102]. Currently, we explore a more efficient synthesis for the JA-Ile-lactone (**7**) and other 12-modified jasmonates based in the metathesis of alkenes employing different Grubbs catalysts (Scheme 3). Preliminary results are very promising.



Scheme 3. Synthetic route to *Z*-enriched 12-modified jasmonates and JA-Ile-lactones. The syntheses of 12-modified jasmonates and JA-Ile-lactones can be reduced to one and three major steps respectively. Reagents and reaction conditions: (a) screening of different protecting groups, solvents and Grubbs type of catalysts to achieve the highest yield of the *Z*-isomer; (b) employing the acetyl group (Ac) as protecting group, the deprotection and saponification can be achieved simultaneously; (c) conjugation of the JA moiety to L-Ile or any other amino acid; (d) macrolactonization is well stablished in our previous work [86]; (f) esterification of JA-Ile with 3-buten-1-ol provides the intermediate to accomplish the RCM as the final step to JA-Ile-lactones; (g) screening of different solvents and Grubbs type of catalysts to achieve the highest yield of the *Z*-isomer of the lactones.

The compounds generated in that way will be employed to investigate both their perception and signaling mechanisms, and their biological activities in different plant species. Understanding the mode of action of the new synthetic jasmonates derivatives will shed light on the JA-signaling pathway and on the potential utilization of these compounds as crop protecting agents.

Methods

General material and methods

All chemicals were obtained from commercial suppliers. If necessary, solvents were purified prior to use [103]. Thin-layer chromatography was performed on silica gel 60 F^{254} on aluminum plates (Merck) and visualized with potassium permanganate staining or phosphomolybdic acid in ethanol. Flash

chromatography was performed on silica gel 60 (40-63 µm) from Merck. Proportions of the employed solvents are referred to volume (v/v) if not mentioned otherwise.

GC-MS spectra were recorded on a ThermoQuest CE Instruments GC 2000 Series coupled to a ThermoQuest Finnigan Trace MS mass spectrometer; GC capillary column HP-5MS (15 m × 0.25 mm ID with 0.25 µm film thickness, Phenomenex). Injection port: 250 °C; Split flow: 15 ml min^{-1} with split ratio of 10 ml min^{-1}; Temperature program: 60 °C (2 min) at 15 °C min^{-1} to 280 °C (5 min). Helium at 1.5 ml min^{-1} served as carrier gas. The ionization method was electron impact (70 eV) in positive mode (EI^+). GC-MS for control of the chemical reactions was carried out in a Hewlett Packard Series II equipment, with a Phenomenex Zebron ZB-5ms (30 m x 0.25 mm, 0.25 *µ*m) column inside and GC conditions as described above for the Trace MS, but in split-less mode. HRMS (ESI^+) was performed in a Bruker Daltonics-maXis Ultra High ResolutionTOF equipment.

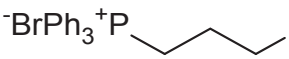
NMR spectra were recorded at 300K either on a Bruker DRX500 spectrometer (frequency 500 MHz for ^1H and 125 MHz for ^{13}C) or a Bruker Avance 400 NMR spectrometer (frequency 400 MHz for ^1H and 100 MHz for ^{13}C). The residual signal of the solvent was used as reference for the chemical shifts. Ozone was generated with a Sander Labor-Ozonisator, oxygen flow at 120 L hr^{-1}, 0.44 A, 90% power at 20 °C.

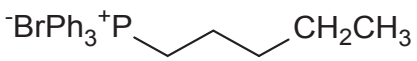
Synthesis of chemicals

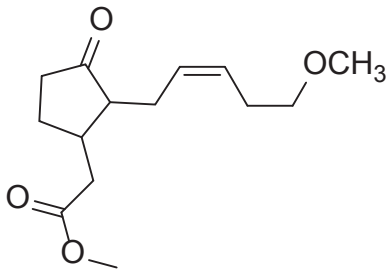
*N-jasmonoyl-L-isoleucine, JA-Ile (**2**):* JA-Ile (**2**) was prepared as previously described in [104].

*Methyl 2-(3-oxo-2-(2-oxoethyl)cyclopentyl)acetate (**8**), 2-(3-bromopropoxy)tetrahydro-2H-pyran (**11**), triphenyl(3-((tetrahydro-2H-pyran-2-yl)oxy)propyl)phosphonium bromide (**12**), methyl (Z)-2-(3-oxo-2-(5-((tetrahydro-2H-pyran-2-yl)oxy)pent-2-en-1-yl)cyclopentyl)acetate (**8a**), (Z)-2-(3-oxo-2-(5-((tetrahydro-2H-pyran-2-yl)oxy) pent-2-en-1-yl)cyclopentyl)acetic acid (**9a**), (2-(3-oxo-2-((Z)-5-((tetrahydro-2H-pyran-2-yl)oxy)pent-2-en-1-yl)cyclopentyl acetyl)-L-isoleucine (**10a**), (2-(2-((Z)-5-hydroxypent-2-en-1-yl)-3-oxocyclopentyl)-acetyl)-L-isoleucine, 12-OH-JA-Ile (**3**), and (4S,Z)-4-((S)-sec-butyl)-3,4,8,11,11a,13,14,14a-*

*octahydro-1H-cyclopenta[g][1]oxa[4]azacy-clotridecine-2,5,12(7H)-trione, JA-Ile-lactone (**7**):* The synthesis of these compounds was carried out as previously described [86].

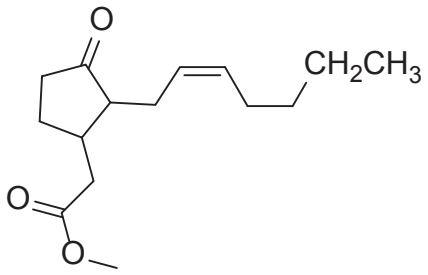
*(3-methoxypropyl)triphenylphosphonium bromide (**13**):*  Commercially available 1-bromo-3-methoxypropane (3.01 g, 20.61 mmol) was dissolved in 20 ml of benzene, after triphenylphosphine (6.49 g, 24.73 mmol) was added and the mixture was refluxed overnight (ca. 15 h). The mixture was worked up the same way as described for compound **12**. Product **13** (7.87 g, 92 %) was kept in a desiccator over $CaCl_2$ until used. ^{1}H NMR (Acetone, 400MHz): δ = 7.96-8.03 (m, 6H), 7.89-7.96 (m, 3H), 7.77-7.84 (m, 6H), 3.75-3.91 (m, 2H), 3.58 (t, *J*=5.9 Hz, 2H), 3.24 (s, 3H), 1.89-2.01 ppm (m, 2H); ^{13}C NMR (Acetone, 100MHz): δ = 135.9, 134.8, 131.3, 120.2, 71.8, 58.6, 23.8, 19.6 ppm

*Pentyltriphenylphosphonium bromide (**14**):*  Commercially available 1-bromopentane (6.00 g, 39.70 mmol) was dissolved in 80 ml of toluene, after triphenylphosphine (12.50 g, 33.08 mmol) was added and the mixture was refluxed overnight (ca. 15 h). The mixture was worked up the same way as described for compound **12**. Product **14** (14.77 g, 90 %) was kept in a desiccator over $CaCl_2$ until used. ^{1}H NMR (Acetone, 500MHz): δ = 8.01-8.07 (m, 6H), 7.87-7.95 (m, 3H), 7.76-7.82 (m, 6H), 3.87-3.95 (m, 2H), 1.65-1.77 (m, 2H), 1.55-1.64 (m, 2H), 1.34 (dq, *J*=14.9, 7.4 Hz, 2H), 0.82 ppm (t, *J*=7.3 Hz, 3H); ^{13}C NMR (Acetone, 125MHz): δ = 135.8, 135.0, 131.1, 120.3, 119.6, 33.2, 33.1, 22.9, 22.7, 22.2, 14.1 ppm

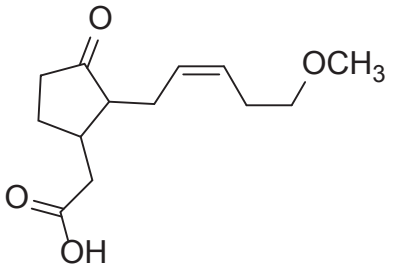
*Methyl (Z)-2-(2-(5-methoxypent-2-en-1-yl)-3-oxocyclopentyl)acetate (**8b**):*  A similar procedure as described for **8a** was followed. **13** (1.65 g, 4.00 mmol, 1.1 eq.), 4.3 ml of KHMDS (0.9 M in THF, 4.4 mmol, 1.2 eq.) and 40 ml of THF were mixed to reach a final concentration of the ylide of approximately 0.2 M. The mixture was stirred at room temperature for 15 min and then cooled down to -78 °C. 0.71 g (3.6 mmol) of **8** dissolved in 16 ml of dry THF were added dropwise and the reaction stirred for an additional hour at -78 °C and another hour after removing the cooling bath. The mixture was filtered through

a fritted funnel and 50 ml of *n*-hexane were added, the precipitate was filtered off and the residual solids were washed twice with 25 ml of *n*-hexane. Evaporation of the solvents and purification with flash chromatography (AcOEt/*n*-hexane, 1:1, R_f 0.34) provided **8b** (0.26 g, 28 %) as a colorless oil. GC-MS (EI^+): 41.10(12%), 45.05(100%), 79.15(10%), 149.15(28%), 222.15(8%), 254.15(7%, M^+); ^{1}H NMR ($CDCl_3$, 500MHz): δ = 5.48 (m, 1H), 5.42 (m, 1H), 3.70 (s, 3H), 3.39 (t, *J*=6.8 Hz, 2H), 3.33 (s, 3H), 2.67-2.76 (m, 1H), 2.20-2.43 (m, 8H), 2.04-2.18 (m, 1H), 1.86-1.96 (m, 1H), 1.43-1.55 ppm (m, 1H); ^{13}C NMR ($CDCl_3$, 125MHz): δ = 218.7, 172.5, 128.2, 127.7, 72.1, 58.6, 53.9, 51.6, 38.7, 38.0, 37.7, 27.9, 27.2, 25.6 ppm

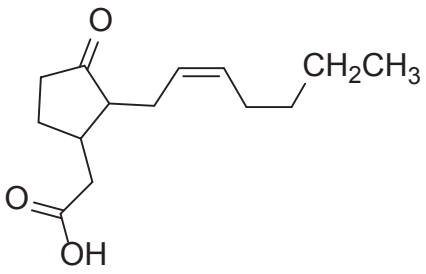
*Methyl (Z)-2-(2-(hept-2-en-1-yl)-3-oxocyclo pentyl)acetate (**8c**):* **14** (1.20 g, 2.91 mmol, 1.1 eq.), 4.6 ml of KHMDS (0.7 M in toluene, 4.4 mmol, 1.2 eq.) and 10 ml of THF were mixed under argon atmosphere to reach a final concentration of the ylide of ca. 0.2 M. The mixture was stirred at room temperature for 15 min and then cooled down to -78 °C. 0.53 g (2.65 mmol) of **8** dissolved in 5 ml of dry THF were added dropwise and the mixture stirred for an additional hour at -78 °C and another hour after removing the cooling bath. The mixture was filtered through a fritted funnel and 30 ml of *n*-hexane were added, the precipitate was filtered off and the residual solids were washed twice with 15 ml of *n*-hexane. Evaporation of the solvents provided crude **8c**, which was employed in the next step without previous purification.



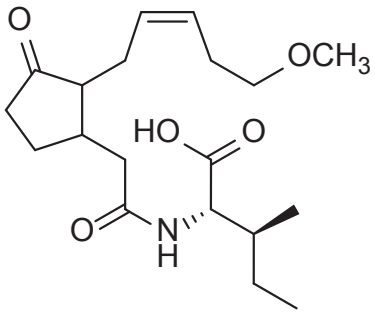
*(Z)-2-(2-(5-methoxypent-2-en-1-yl)-3-oxocyclopentyl)acetic acid (**9b**):* **8b** (0.26 g, 1.02 mmol) was dissolved in 10 ml of MeOH and 15 ml of NaOH (1 M) were added. The reaction was heated at 70 °C for 1 h and diluted after with 25 ml of H_2O. A solution of HCl (1 M) was employed to adjust the pH to around 3. Aqueous phase was then extracted three times with portions of AcOEt (20 ml). The combined organic extracts were washed with brine and dried over anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure afforded **9b** (0.24 g, 96 %), which was employed in the next reaction without further purification.

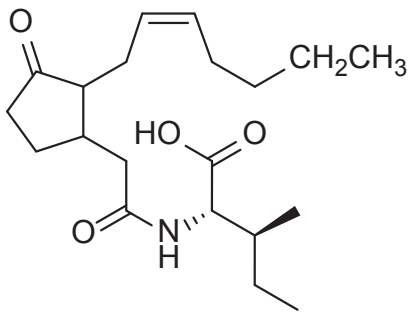


*(Z)-2-(2-(hept-2-en-1-yl)-3-oxocyclopentyl)acetic acid (**9c**):* The crude product obtained in the synthesis of **8c** was dissolved in 5 ml of MeOH and after 25 ml of NaOH (0.5 M) were added. The mixture was heated at 85 °C for 1 h and diluted after with 25 ml of H_2O. A solution of HCl (1 M) was employed to adjust the pH to around 3. Aqueous phase was then extracted three times with portions of AcOEt (25 ml). The combined organic extracts were washed with brine and dried over anhydrous $MgSO_4$. Evaporation of the solvent under reduced pressure afforded **9c** (0.11 g, 17.4 % from aldehyde **8**), which was employed in the next reaction without further purification.



*(2-(2-((Z)-5-methoxypent-2-en-1-yl)-3-oxocyclopentyl)acetyl)-L-isoleucine, 12-OCH3-JA-Ile (**5**):* The conjugation of L-Ile to **9b** was carried out as previously described for **10a**. Crude **9b** (0.10 g, 0.43 mmol) and triethylamine (48 mg, 0.47 mmol, ~ 1.1 eq.) were dissolved in THF (12 ml) and after ECF (50 mg, 0.46 mmol, ~ 1.1 eq.) was added under stirring at -5 °C. After 5 min L-Ile (0.11 g, 0.86 mmol, ~ 2 eq.) dissolved in aqueous NaOH (5 ml, 0.3 M) was added and stirring was continued for 30 min at room temperature. The reaction mixture was then acidified with HCl (1 M) and extracted with AcOEt. The combined organic extracts were dried over $MgSO_4$ and after removal of solvents, flash chromatography ($CHCl_3$/AcOEt/AcOH, 14:6:1) afforded 12-OCH_3-JA-Ile (**5**) (101.8 mg, 67 %). Silica gel TLC R_f = 0.16; GC-MS (EI^+) **5**-methyl ester: 44.95(100%), 86.00(65%), 128.00(56%), 146.05(25%), 367.10(8%, M^+); HRMS (ESI^+-TOF): m/z = 352.2129 $[M-H]^+$ (calc. for $C_{19}H_{30}NO_5$, 352.2124); ^1H NMR ($CDCl_3$, 500MHz): δ = 6.33 (d, *J*=8.3 Hz, 1H), 5.37-5.51 (m, 2H), 4.59 (ddd, *J*=8.3, 5.1, 2.2 Hz, 1H), 3.43 (td, *J*=6.6, 3.4 Hz, 2H), 3.35 (s, 3H), 2.50-2.71 (m, 1H), 2.17-2.44 (m, 8H), 2.03-2.16 (m, 1H), 1.86-2.01 (m, 2H), 1.45-1.61 (m, 2H), 1.14-1.29 (m, 1H), 0.90-0.99 ppm (m, 6H); ^{13}C NMR ($CDCl_3$, 125MHz): δ = 219.4, 175.0, 171.9, 128.3, 128.1, 72.4, 58.6, 56.6, 54.3, 41.2, 38.8, 37.5, 27.8, 27.3, 27.1, 25.9, 25.3, 15.7, 11.7 ppm





*(2-(2-((Z)-hept-2-en-1-yl)-3-oxocyclopentyl)acetyl)-L-isoleucine, 7-hept-JA-Ile (**6**):* **9c** (0.110 g, 0.46 mmol) and triethylamine (51 mg, 0.5 mmol, ~ 1.1 eq.) were dissolved in THF (12 ml) and after ECF (54 mg, 0.5 mmol, ~ 1.1 eq.) was added under stirring at ca. -5 °C. After 5 min, L-Ile (0.121 g, 0.92 mmol, ~ 2 eq.) dissolved in aqueous NaOH (5 ml, 0.3 M) was added and stirring was continued for 30 min at room temperature. The reaction mixture was acidified with HCl (1 M) and extracted with AcOEt. The combined organic extracts were dried over $MgSO_4$ and after removal of the solvent, flash chromatography ($CHCl_3$/AcOEt/AcOH, 30:15:1) afforded 7-hept-JA-Ile (**6**) (72 mg, 45 %,). Silica gel TLC R_f = 0.2; GC-MS (EI^+) **6**-methyl ester: 40.95(55%), 54.90(48%), 86.00(100%), 128.10(73%), 146.05(58%), 187.05(24%), 347.00(12%), 365.15(16%, M^+); HRMS (ESI^+-TOF): m/z = 350.2337 $[M]^+$ (calc. for $C_{20}H_{32}NO_4$, 350.2331); ^1H NMR ($CDCl_3$, 500MHz): δ = 6.15 (d, *J*=8.2 Hz, 1H), 5.40-5.51 (m, 1H), 5.25-5.36 (m, 1H), 4.66 (dd, *J*=8.3, 4.6 Hz, 1H), 2.69 (dd, *J*=14.0, 4.0 Hz, 1H), 2.28-2.45 (m, 4H), 2.08-2.28 (m, 3H), 1.86-2.08 (m, 4H), 1.44-1.60 (m, 2H), 1.14-1.38 (m, 6H), 0.93-0.99 (m, 6H), 0.84-0.92 ppm (m, 3H); ^{13}C NMR ($CDCl_3$, 125MHz): δ = 219.7, 175.7, 171.8, 132.6, 125.8, 56.5, 54.3, 41.4, 38.8, 37.8, 37.8, 31.9, 27.2, 27.2, 25.9, 25.2, 22.5, 15.7, 14.1, 11.7 ppm

Plant treatments

A. thaliana seeds (ecotype Columbia) were used in the experiments. Seeds were sown in 10 cm round pots and stratified for 3-4 days at 4°C. Afterwards plants were moved to ventilated growth rooms with constant air flow, 57-71% humidity and 21°C. Plants were grown at a distance of 30 cm from fluorescent light banks with 6 bulbs of cool white and 2 bulbs of wide spectrum lights at a 10 h light/14 h dark photoperiod and a light intensity of 90-130 µmol m^{-2} s^{-1}. The plants were shifted to wide spectrum light source 3 weeks after growth and all the experiments were performed on 5-week-old plants. All induction experiments were performed 4 weeks post germination at a vegetative (pre-bolting) growth stage. JA-Ile and derivatives were prepared as stock solutions of 50 mM in 100% ethanol. A solution of JA-Ile or the analogue (200 µM) was sprayed onto plants and incubated for 16

hours before gene expression studies. A solution of 0.4% ethanol was used as solvent control.

Expression analysis by RT-PCR

Leaf material was ground to a fine powder in liquid N_2 and total RNA was isolated by means of the TRIzol Reagent (Invitrogen) according to the protocol provided by the manufacturer. An additional DNAse (Turbo DNAse, Ambion) treatment was included to eliminate any contaminating DNA. RNA quantity was determined photospectrometrically. DNA-free total RNA (1 µg) was converted into single-stranded cDNA using a mix of oligo-dT_{20} primers and the Omniscript cDNA synthesis kit (Qiagen). Gene-specific primers (placed at the exon-exon junction for specific amplification of cDNA, whenever possible) were designed with the help of the NCBI primer design tool (http://www.ncbi.nlm.nih.gov). Primers producing 150 to 170 bp amplicons were used for real time PCR. Q-RT-PCR was carried out in optical 96-well plates on a CFX 96 Real-Time PCR Detection System (Biorad) employing the Brilliant QPCR SYBR green Mix (Agilent) to monitor double-stranded DNA synthesis. *RPS18B* performed best as an endogenous control ('normalizer') upon herbivory. Thus, the mRNA levels for each cDNA probe were normalized with respect to the *RPS18B* mRNA level. Fold induction values of target genes were calculated with the ΔΔCP equation [105] and related to the mRNA level of target genes in control leaf, which were defined as 1. All of the assays were run in triplicate (biological replication) to control for overall variability. The primer pairs used are listed below:

RPS18B (*At1g 34030*)	F-5'- GTCTCCAATGCCCTTGACAT -3'
	R- 5'-TCTTTCCTCTGCGACCAGTT -3'
AOS (At5g42650)	F-5'-AAGCCACGACGCGGCGTTTA-3'
	R- 5'-GGAGTCTCCGTCTCCGGTCCA-3'
MYC2 (At1g 32640)	F-5'- CGGAGATCGAGTTCGCCGCC -3'
	R- 5'- AATCCCGCACCGCAAGCGAA-3'

THI2.1 (At1g72260) F-5'- CGCCATTCTCGAAAACTCAGCTGA -3'

R- 5'- GTTTAGGCGGCCCAGGTGGG -3'

VSP2 (At5g24770) F- 5'- ACGACTCCAAAACCGTGTGCAA -3'

R- 5'- CGGGTCGGTCTTCTCTGTTCCGT-3'

PDF1.2 (AT5G44420) F- 5'- CTGCTTTCGACGCACCGGCA-3'

R- 5'- GTTGCATGATCCATGTTTGGCTCCT-3'

COI1 (AT2G39940) F- 5'- TCGCTTTGGCTCAGGGCTGC-3'

R- 5'- CGGACTCCGTTGTCCAGTGGC-3'

CYP94B3 (AT3G48520) F- 5'- ATGGACCACCATCGTATCCACTCA-3'

R- 5'- GCGACGGTTGCCGAGGAGAG-3'

Supplementary Information

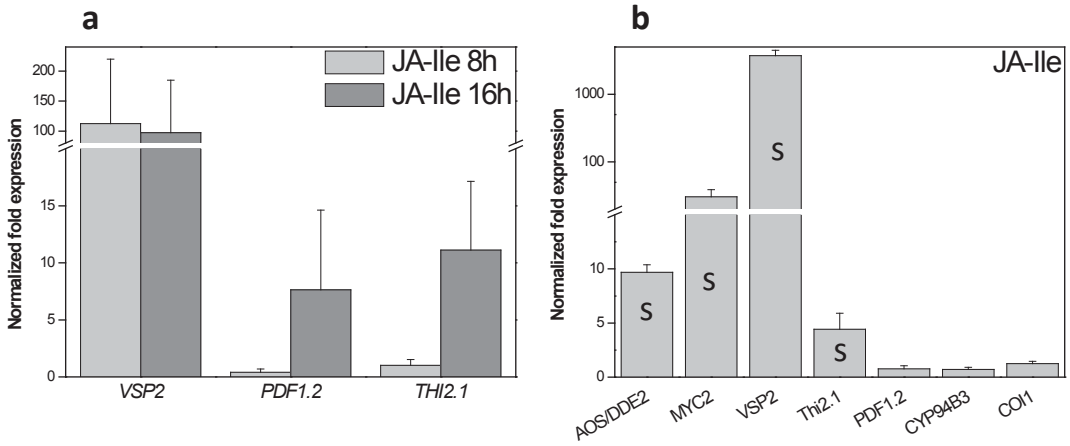
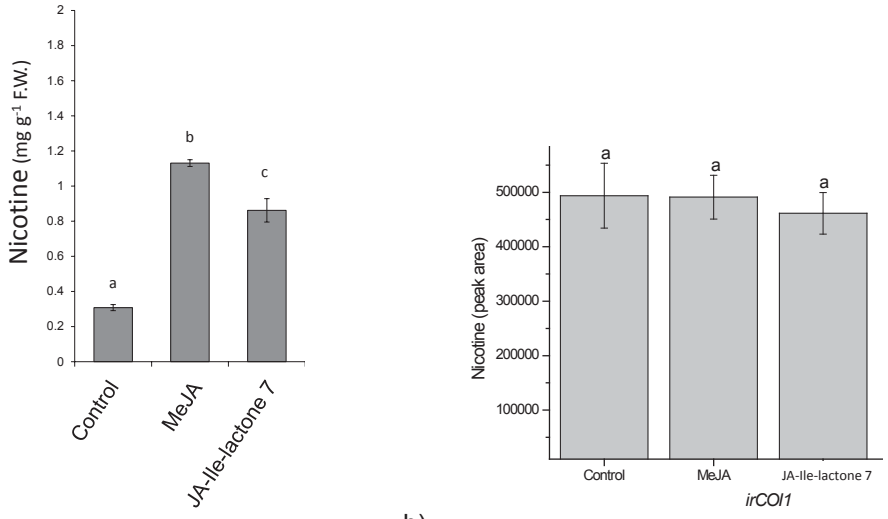


Figure S1: a) Expression levels of JRGs at 8 and 16 h after spraying the plant leaves with JA-Ile (**2**). **b)** Expression levels of several JRGs after 16 h. Genes selected as markers are labelled with "S".

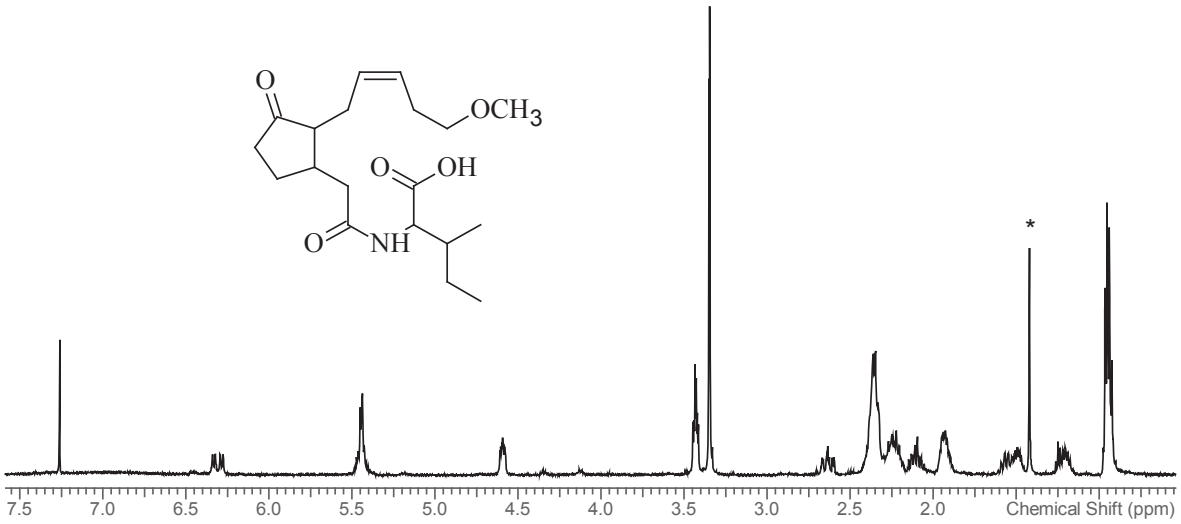


a) b)

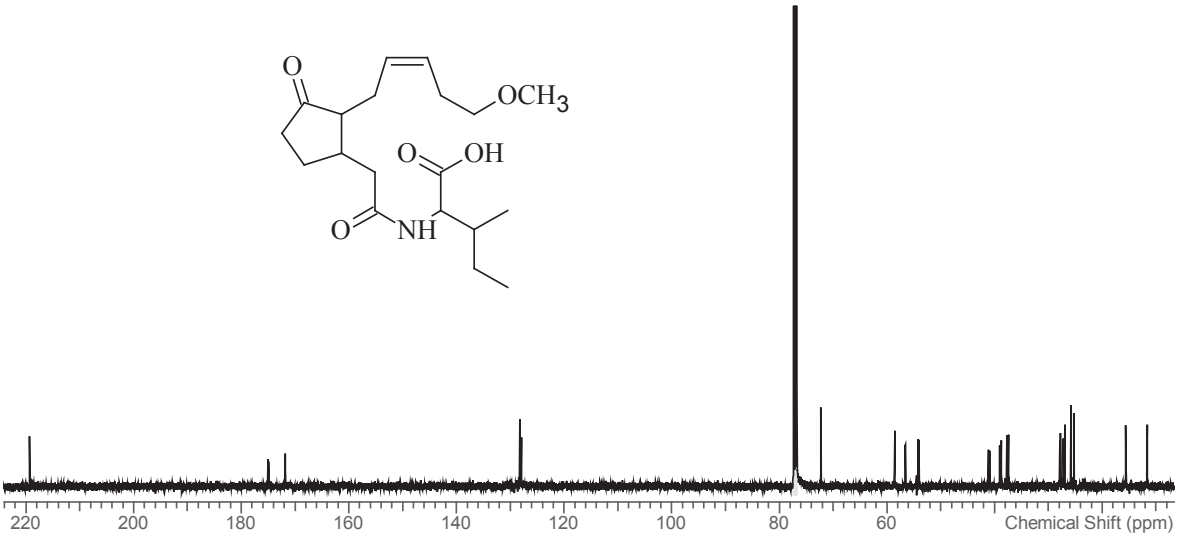
Figure S2: Nicotine induction by JA-Ile-lactone (**7**) occurs in a NaCOI1 dependent manner; a) JA-Ile-lactone (**7**) induces nicotine accumulation in WT plants; b) the nicotine accumulation is not observed in ir-*COI1* silenced lines.

Copy of NMR spectra of the new compounds (5) and (6)

- 12-OCH_3-JA-Ile (**5**)



^1H NMR ($CDCl_3$, 500MHz). * Cyclohexane.



^{13}C NMR ($CDCl_3$, 125MHz)

Mass Spectrum SmartFormula Report

Analysis Info

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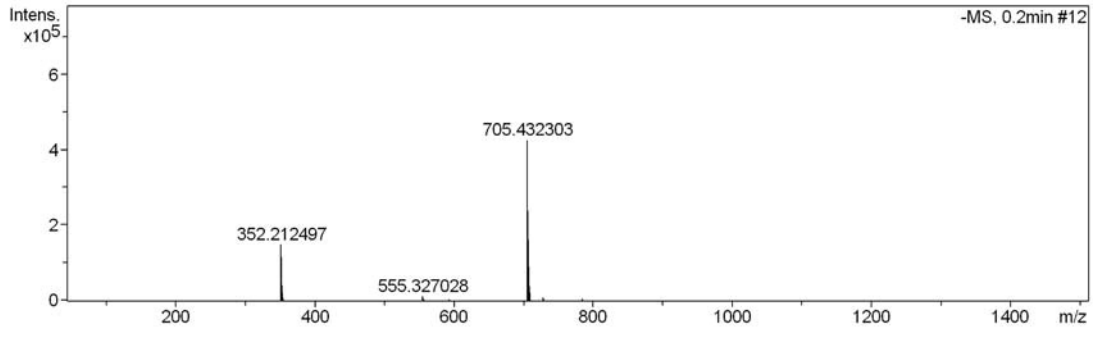
Method	kp_tune_low_neg_250_JA.m	Operator	Admin
Sample Name	12 -OMe	Instrument / Ser#	maXis 10078
Comment			

Acquisition Parameter

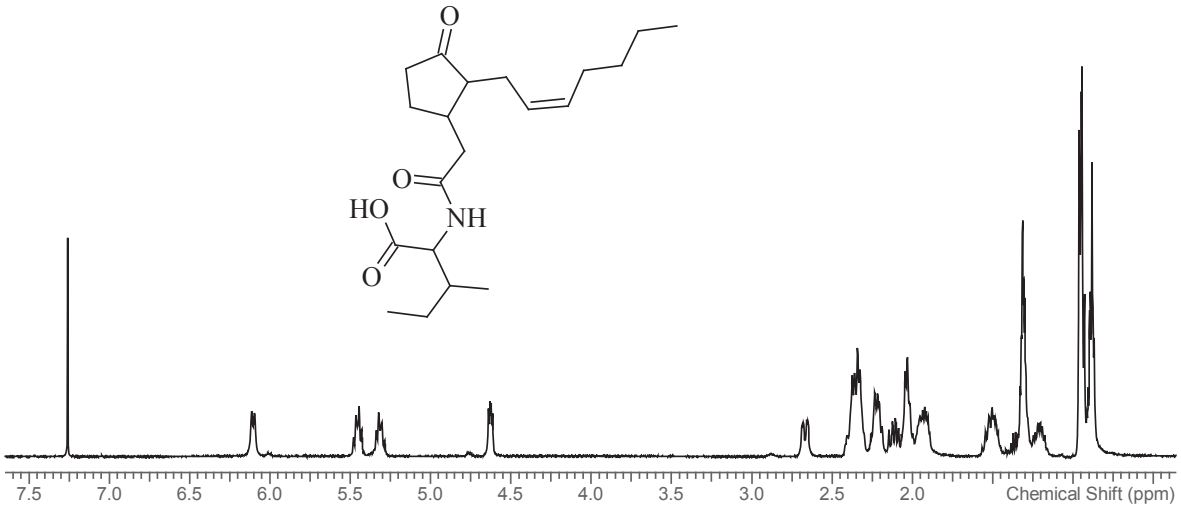
Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	3000 V	Set Dry Heater	190 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	1000.0 Vpp	Set Divert Valve	Waste



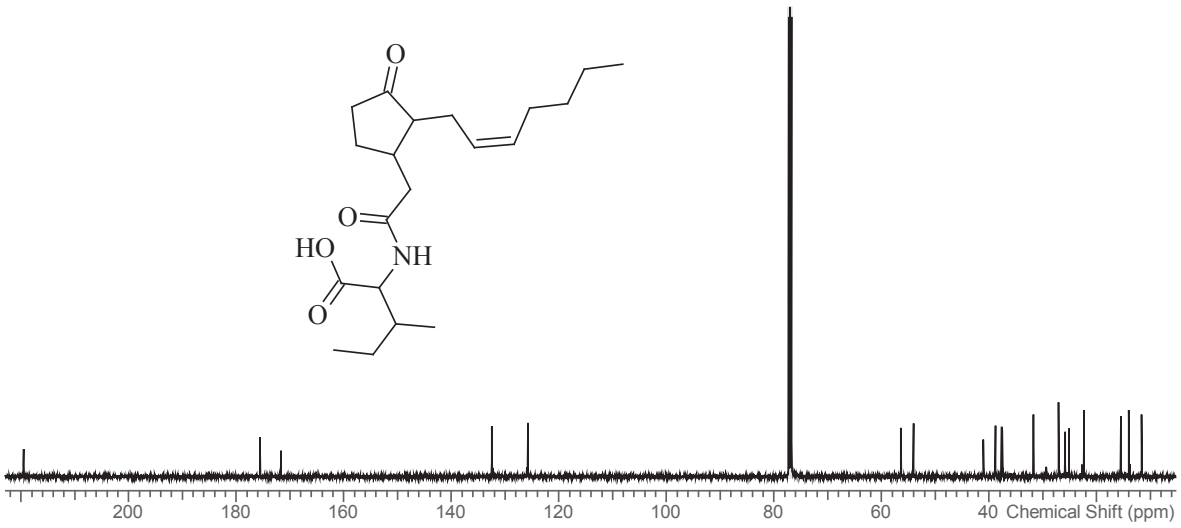
Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
352.212497	1	C 19 H 30 N O 5	100.00	352.212947	0.450	1.278	9.0	5.5	even	ok

HRMS

- 7-hept-JA-Ile (**6**)



^{1}H NMR ($CDCl_3$, 500MHz)



^{13}C NMR ($CDCl_3$, 125MHz)

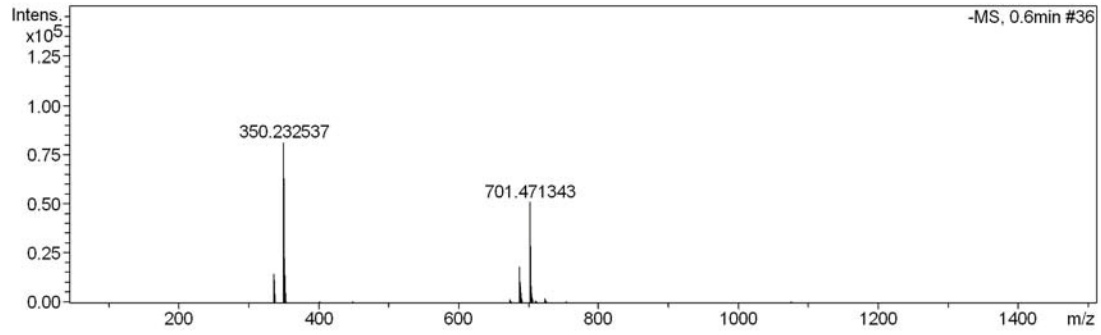
Mass Spectrum SmartFormula Report

Analysis Info

Analysis Name	D:\Data\kepl2401\Guillermo_12_Et_Messg4.d	Acquisition Date	12/11/2014 8:33:32 PM
Method	kp_tune_low_neg_250_JA.m	Operator	Admin
Sample Name	12 - Et-	Instrument / Ser#	maXis 10078
Comment			

Acquisition Parameter

Source Type	ESI	Ion Polarity	Negative	Set Nebulizer	0.4 Bar
Focus	Not active	Set Capillary	3000 V	Set Dry Heater	190 °C
Scan Begin	50 m/z	Set End Plate Offset	-500 V	Set Dry Gas	4.0 l/min
Scan End	1500 m/z	Set Collision Cell RF	1000.0 Vpp	Set Divert Valve	Waste



Meas. m/z	#	Formula	Score	m/z	err [mDa]	err [ppm]	mSigma	rdb	e⁻ Conf	N-Rule
350.232537	1	C 20 H 32 N O 4	100.00	350.233682	1.145	3.268	9.1	5.5	even	ok
	2	C 16 H 28 N 7 O 2	64.84	350.230997	-1.541	-4.399	18.4	6.5	even	ok

HRMS

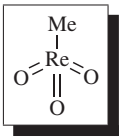
8. Article IV

Methyltrioxorhenium (VII). Second Update

Guillermo H. Jimenez-Aleman and Wilhelm Boland.

e-EROS. Encyclopedia of Reagents for Organic Synthesis 2013, pp. 12-17. Wiley.
DOI: 10.1002/047084289X.rn00017.pub3

Methyltrioxorhenium



[70197-13-6] CH_3O_3Re (MW 250)
InChI = 1S/CH3.3O.Re/h1H3;;;;
InChIKey = PQTLALPZRPFYIT-UHFFFAOYSA-N

(catalyst in the hydrogen peroxide oxidation of numerous compounds, including the well-studied epoxidation of alkenes.[5] Catalyzes 1,3-allylic transpositions,[6] the metathesis of alkenes[7,8] and aldehyde olefination[9,10])

Physical Data: thermally stable white solid, mp 111 °C. Hydrolyzed rapidly in basic aqueous solutions but much slower in acidic solutions. Deactivated under photolysis conditions.

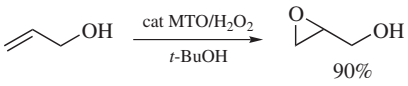
Solubility: highly soluble in virtually any solvent from pentane to water.

Form Supplied in: commercially available from Aldrich and Fluka.

Original Commentary

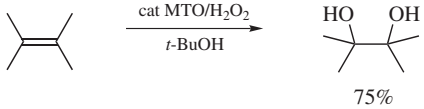
Andrew Hudson
Wayne State University, Detroit, Michigan, USA

Epoxidation of Alkenes.[5] In 1991, Hermann demonstrated that alkenes are efficiently converted to the corresponding epoxide using methyltrioxorhenium (MTO) as the catalyst and hydrogen peroxide as the co-oxidant.[11] A range of alkenes undergo epoxidation at ambient temperatures with a catalyst loading of 0.1 to 1 mol % (eq 1).



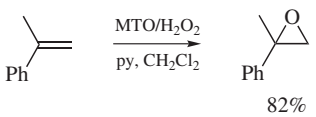
(1)

However, serious limitations of the original procedure included ring opening or oxidative cleavage of the newly formed epoxide, a consequence of the acidic nature of the rhenium catalyst (eq 2).

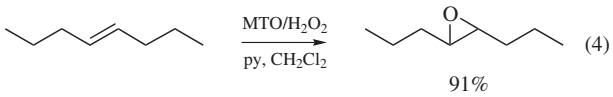


(2)

The addition of tertiary amines, usually pyridine, has been found to be successful in both suppressing such side reactions and substantially increasing the rate of epoxidation.[12] The reaction is remarkably general for efficient epoxidation of a number of alkenes (eqs 3–6).



(3)



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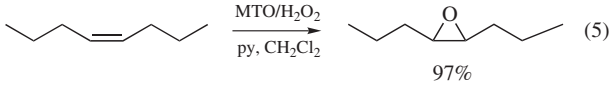

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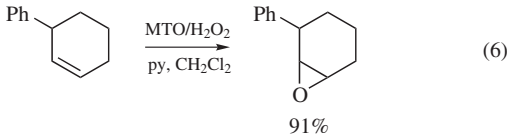
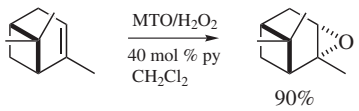


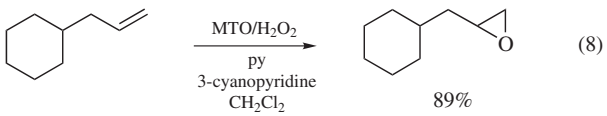
(6)

It must be stressed that the amount of amine added to the MTO-catalyzed epoxidation reaction is critical. The addition of tertiary amines was found to significantly inhibit catalyst activity at low concentrations. Studies have shown that the lifetime of the catalyst is intrinsically linked to the amount of pyridine present and concentrations of 3 mol % or more of pyridine in nitromethane or dichloromethane are required for high conversions of alkenes. An example that highlights this not well-understood phenomena is the epoxidation of monoterpenes (eq 7).[13] A relatively high mol % of pyridine had to be employed in order to produce high yields. Even higher mol % of pyridine significantly decreased the yield. The tertiary amine additives are thought to play a crucial role in preventing decomposition of the epoxide products, prolonging the lifetime of the catalyst in solution and increasing the rate of epoxidation. An extensive amount of work on the equilibria and kinetics of amine additives in MTO-catalyzed epoxidation reactions has been undertaken.[14]



(7)

Other tertiary amines have been utilized in the MTO-catalyzed epoxidation reactions. Pyrazole was also found to be an effective additive for a variety of alkenes.[15] The use of an equimolar amount of 3-cyanopyridine and pyridine as an additive for the epoxidation of terminal alkenes has been found to be high yielding with little-to-no destruction of the resulting epoxide detected.[16,17] This system is effective for the epoxidation of a range of alkenes, in particular alkenes of relatively low reactivity (eqs 8–10). Electron deficient alkenes did not perform well under such epoxidation conditions.



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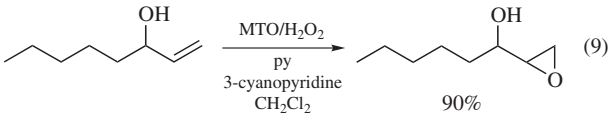

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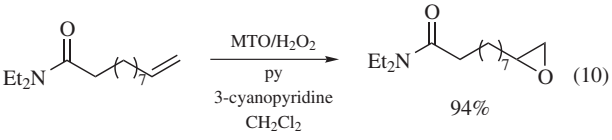
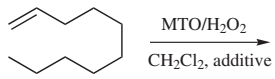
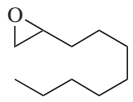


(10)

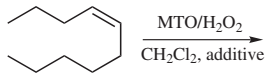
A comparative study of the effectiveness of various amine additives has been undertaken (eqs 11–13).[18]

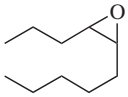




Additive
py 60%
3-cyanopyridine 99%
pyrazole 99%

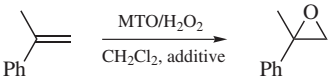
(11)





Additive
py 99%
3-cyanopyridine 60%
pyrazole 99%

(12)

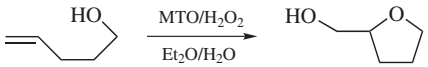


Additive
py 53%
3-cyanopyridine 74%
pyrazole 93%

(13)

Although pyridine is less expensive than pyrazole, the latter seems to be more general. However, the choice of amine additive remains unclear when applying this methodology to new substrates and, although somewhat predictable, may be a matter of experimental trial and error.

Treatment of homo-allylic alcohols with catalytic MTO and hydrogen peroxide results in epoxidation followed by hydroxyl lactonization to yield substituted tetrahydrofurans in high yield (eq 14).[19] Alcohols, acids and esters all undergo such domino epoxidation/cyclization reactions.[20]



(14)

The epoxidation process has several distinct advantages over existing methodology. The epoxidation is carried out under neutral (non-acidic) conditions and does not suffer from unwanted side reactions. All the reagents used in the MTO-catalyzed epoxidation are easily handled and commercially available. In comparison, *meta*-chloroperbenzoic acid (*m*-CPBA) is only available as a mixture with the acid and cannot be purified safely due to its explosive nature. DMDO needs to be prepared and titrated directly prior to use.

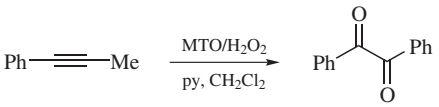
Typical Procedure for the Epoxidation of Alkenes.[17] The alkene (10 mmol) was dissolved in dichloromethane (3 mL, 2 M concentration in alkene) to which the amine (1.2 mmol) and MTO (0.05 mmol) was added. Then 30% hydrogen peroxide (20 mmol) was added and the reaction vigorously stirred whereupon the solution turns yellow, indicative of the formation of the active catalyst. After 1h a catalytic amount of manganese oxide was added (to destroy any remaining peroxides in solution). When evolution of oxygen had stopped, the layers were separated, the aqueous layer extracted with dichloromethane (3 × 25 mL) and the combined organic extracts dried (Na_2SO_4) and concentrated under reduced pressure.

Other systems have been developed in the formation of acid-sensitive epoxides. Adam has developed an MTO-catalyzed epoxidation process using a urea/hydrogen peroxide (UHP) adduct.[21] Although this methodology allows for the isolation of some acid-sensitive epoxides, other alkenes, such as α-methylstyrene, gave significant amounts of the corresponding 1,2-diols and yields in many cases were only modest.

Sodium percarbonate (the so-called 'solid form' of hydrogen peroxide) has been utilized in the MTO-catalyzed epoxidation.[22] Although the use of sodium percarbonate offers no improvement in performance, it uses a safer and more easily handled co-oxidant that may prove to have application on an industrial scale. Trifluoroethanol has been used as the solvent in the MTO-catalyzed reaction of alkenes and has shown enhanced rates of epoxidation for a variety of alkenes at low catalyst loading (0.1 mol %).[23] Unfortunately the lack of solubility of non-polar alkenes in such a fluorinated solvent remains a problem. Epoxidation of alkenes in an ionic liquid has been reported and the use of no organic solvents has possible industrial applications in a more eco-friendly process.[24] A limitation of this process is once again the low solubility of certain alkenes in an ionic liquid. For example, 1-decene showed poor solubility in such a system which manifested itself in a modest yield of 46%. MTO has been supported on a silica tether allowing for epoxidation of alkenes under environmentally benign conditions.[25] Although such a system has much promise the silica supported MTO has lower reactivity in comparison to its homogeneous partner.

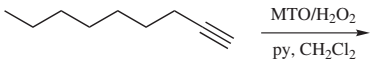
Regio- and Diastereoselectivity. Unlike many other transition metal-catalyzed epoxidation reactions, little-to-no diastereoselectivity is usually detected in the MTO-catalyzed epoxidation of allylic alcohols.[21,26] An extensive comparative study has been undertaken showing that metal alcoholate binding does not apply in MTO-catalyzed epoxidation reactions.[27] This observation tends to suggest that a rhenium peroxo complex is the active oxidant. Computational experiments have demonstrated that the rhenium *bis*(peroxo) complex (probably the hydrated form) is the active species in the MTO-catalyzed epoxidation of propenol.[28]

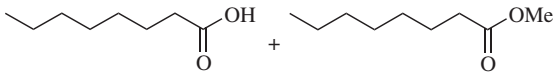
Oxidation of Alkynes. The MTO/hydrogen peroxide oxidation of alkynes has been studied.[29] Internal alkynes yield predominantly diketones (eq 15), whilst terminal alkynes yield the corresponding acid or esters, depending on the solvent employed (eq 16).



79%

(15)



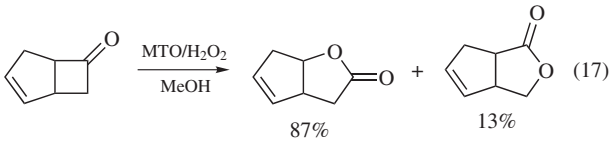


18% 81%

(16)

Baeyer-Villiger Oxidation. Oxidation of cyclic and acyclic ketones to the corresponding lactones and esters has been achieved. For example, cyclobutanone is converted to the

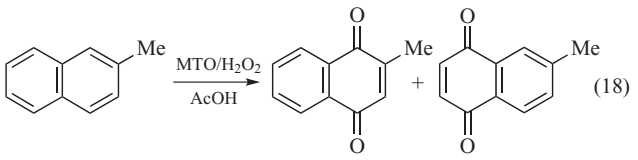
corresponding lactone under the usual MTO/hydrogen peroxide conditions in almost quantitative yield in less than 1 h.[30] The scope of this reaction has been explored.[31] γ-Butyrolactones were obtained in high yield and regioselectively by an MTO-catalyzed hydrogen peroxide Baeyer-Villiger oxidation (eq 17).



(17)

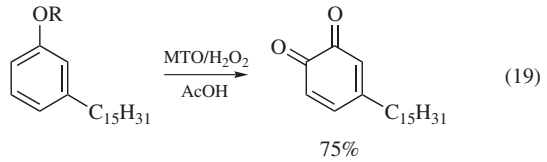
Noteworthy is the chemoselectivity of the process - the oxidation can even be done in the presence of alkene moieties.

Aromatic Oxidation. The oxidation of aromatic groups to quinones is another facet of the MTO-catalyzed oxidation reactions with hydrogen peroxide and is of particular importance in the production of vitamin K_3 (eq 18).[32] A highly acidic solution with a high concentration of hydrogen peroxide is essential. The reaction is both remarkably high yielding and selective.



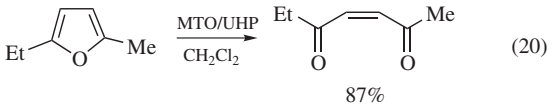
(18)

An efficient synthesis of *ortho*- and *para*-benzoquinones of cardanol derivatives has been described using a similar oxidative process (eq 19).[33] Regioselectivity depends upon the nature and substitution pattern of the aromatic ring.



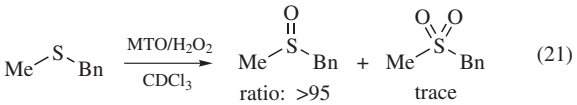
(19)

It has been shown that furan derivatives can be oxidized to the corresponding enediones using MTO and urea/hydrogen peroxide (eq 20).[34] Yields were high in all cases.



(20)

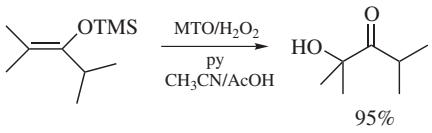
Oxidation of Sulfides. The MTO/hydrogen peroxide system effectively oxidizes sulfides to the corresponding sulfoxides (eq 21).[35,36]



(21)

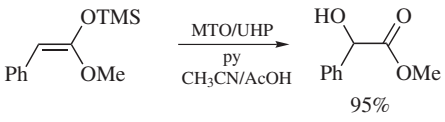
Further oxidation to the sulfone was found to be very slow in comparison to sulfide oxidation, and could be achieved on addition of a further equivalent of hydrogen peroxide. Thioketones have been oxidized in a similar fashion to yield ketones with expulsion of sulfur dioxide.[37] Symmetrical disulfides have been oxidized to the corresponding thiosulfinates, thiosulfonates, and sulfonic acids.[38]

Oxidation of Silyl Compounds. MTO-catalyzed oxidization of silyl enol ethers with hydrogen peroxide yields α-hydroxyketones in high yield.[39] The reactions were conducted with pyridine as the amine additive in acetic acid (eq 22).



(22)

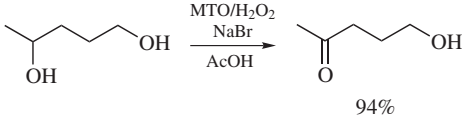
It is thought that the acid lowers the basicity of the solution increasing catalyst lifetime. The addition of acid alone resulted in total hydrolysis of the silyl-enol ether. Methyl trimethylsilyl ketene acetals undergo oxidation using the anhydrous MTO/UHP system,[21] yielding α-hydroxyesters in high yield for a number of substrates (eq 23).[40]



(23)

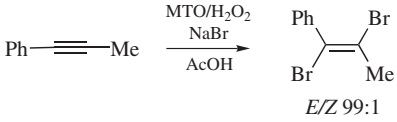
Oxidation of triorganosilanes to silanols has been achieved by treatment with MTO/UHP.[41] High conversions and excellent selectivity over disiloxane products were obtained. Of particular interest is the transformation of an optically active silane to its corresponding silanol with almost complete retention configuration via oxidative insertion.[41]

Oxidation and Bromination of Alcohols. Although the oxidation of alcohols by hydrogen peroxide alone is negligible, catalytic amounts of bromide ions greatly enhance the rate of MTO-catalyzed oxidation of alcohols with hydrogen peroxide.[42] Interestingly, a secondary alcohol has been selectively oxidized in the presence of a primary alcohol (eq 24).[42]



(24)

Under similar conditions, acetylene and phenols were brominated in quantitative yields (eq 25).[42] In the case of the dibromination of acetylene derivatives *E/Z* selectivity was high at high bromide ion concentrations.

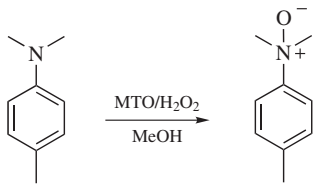


(25)

Oxidation of Phosphines, Arsenides, and Stannanes. Under standard MTO-catalyzed oxidation conditions with hydrogen peroxide, tertiary phosphines, triphenylarsine, and triphenylstibene were all efficiently oxidized.[43] A detailed account of the rates of reactions of these oxidative processes has been reported

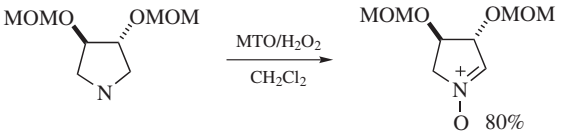
which supports a mechanism that allows for nucleophilic attack of the substrate at the rhenium peroxide species.[43]

Oxidation of Nitrogen-Containing Compounds. Various substituted aniline derivatives have been oxidized under MTO/hydrogen peroxide conditions in good yield to the corresponding *N*-oxide derivatives (eq 26).[44]



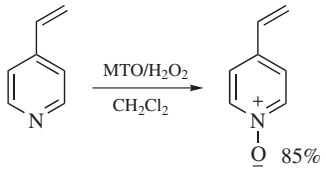
(26)

Nitrones are formed upon MTO/hydrogen peroxide oxidation of secondary amines (eq 27).[45]



(27)

In the same manner, pyridine derivatives are oxidized to their respective *N*-oxides in good yield.[46] Noteworthy is that *N*-oxidation, and not epoxidation, was observed when treating the substrates with MTO/hydrogen peroxide (eq 28); non-conjugated systems under such oxidative systems gave epoxides in preference to *N*-oxidation (eq 29). No degradation of the MTO catalyst was seen on *N*-oxidation, an observation that is not paralleled in the MTO-catalyzed epoxidation of alkenes.



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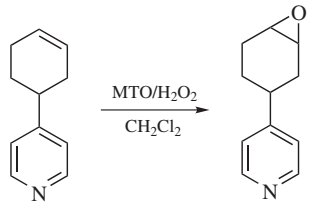
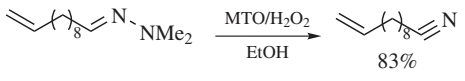


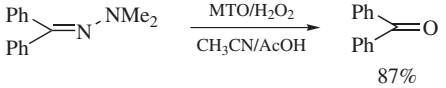
(29)

N,N-Dimethyl hydrazones, derived from aldehydes, are efficiently oxidized to the corresponding nitriles in high yield (eq 30).[47,48] A number of examples have been reported.



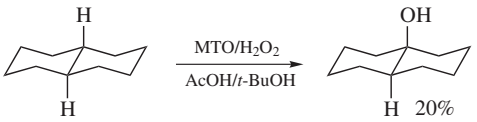
(30)

Interestingly, hydrazones derived from ketones were found to yield the corresponding ketones (eq 31).[49]



(31)

Oxidative Insertion. MTO has been found to catalyze oxidative insertion of remote C–H bonds in the presence of a large excess of hydrogen peroxide.[50] This intriguing observation has been applied to a number of different hydrocarbons. Moreover, the reactions are stereospecific, as exemplified in the case of *cis*- and *trans*-decalin (eqs 32 and 33).



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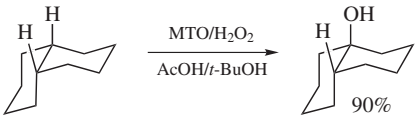
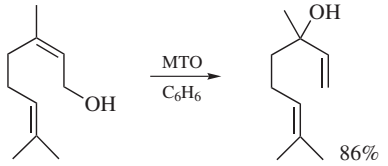


(33)

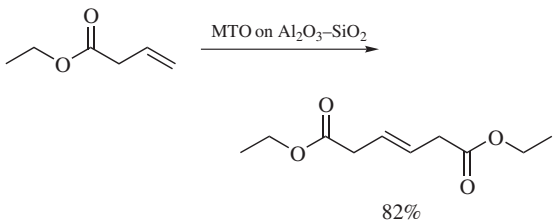
Dehydration, Amination, and Disproportionation of Alcohols. Ether formation, dehydration and disproportionation reactions catalyzed by MTO has been carried out.[51] Yields were found to vary dramatically depending upon the substrate. These reactions have limited synthetic value and at present offer no advantages to existing technologies in each of these areas.

1,3-Allylic Transpositions. MTO is an effective catalyst in the isomerization of allylic alcohols. A variety of allylic alcohols have been tried and yields were generally high (eq 34).[6] Side reactions included condensation and dehydration of the product, processes that have been reported by the same group.[6]



(34)

Metathesis. MTO is active in alkene metathesis when activated by a co-catalyst such as $S_4N_4/AlCl_3$, or supported on silica or alumina.[7,8] In the original communication of Hermann et al.,[7] the self-metathesis of allylic halides, silanes and unsaturated carboxylates and nitriles was achieved using MTO/Al_2O_3-SiO_2 as the catalytic system (eq 35).

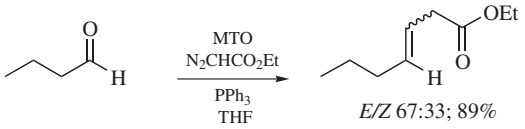


(35)

At present, the metathesis reactivity of MTO has not been developed as a synthetically viable reagent in comparison to the well established Schrock and Grubb catalysts available.

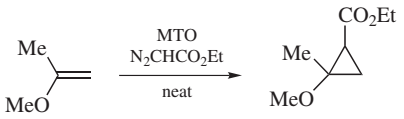
Formation of Alkenes from Aldehydes. Treatment of various aldehydes with diazoalkenes and tertiary phosphines in the presence of MTO gave the corresponding coupled product in

high yield.[9,10] *E/Z* selectivity was low in most cases (eq 36). An obvious advantage over Tebbe-Grubbs-type coupling reactions is that only a catalytic amount of the organometallic coupling reagent is required.

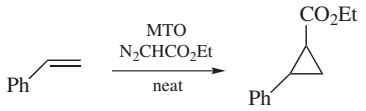


E/Z 67:33; 89% (36)

Cyclopropanation and Aziridination. MTO catalyzes both the reactions of ethyldiazoacetate and organic azides with alkenes and carbonyl compounds, respectively, to yield cyclopropanes in good yield.[52] A number of examples of the cyclopropanation of alkenes have been reported under mild conditions (eqs 37 and 38). The reaction is remarkably general to differing steric and electronic environments of the alkene.

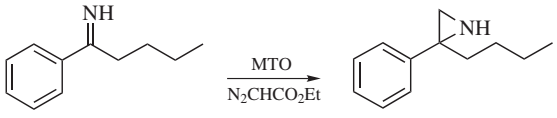


69% (37)



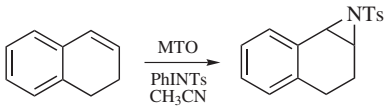
81% (38)

The analogous reaction with imines or carbonyl compounds results in the corresponding aziridines or epoxides respectively (eq 39).[52]



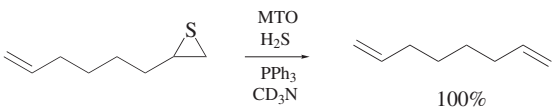
92% (39)

MTO-catalyzed aziridination has also been achieved from alkenes utilizing [*N*-(*p*-tolylsulfonyl)imino]iodobenzene as the nitrene transfer reagent (eq 40).[53] Although yields at present are moderate to poor the reaction holds much promise for effective aziridination of alkenes.



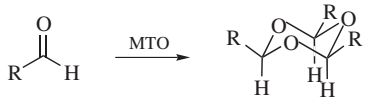
43% (40)

Miscellaneous Reactions. MTO catalyses the formation of alkenes from *epi*-sulfides with triphenylphosphine (eq 41).[54] The reaction is general and high yielding at room temperature.



100% (41)

MTO also catalyzes the trimerization of aldehydes - one of the three oxygen atoms in the product is derived from MTO (eq 42).[55]



(42)

First Update

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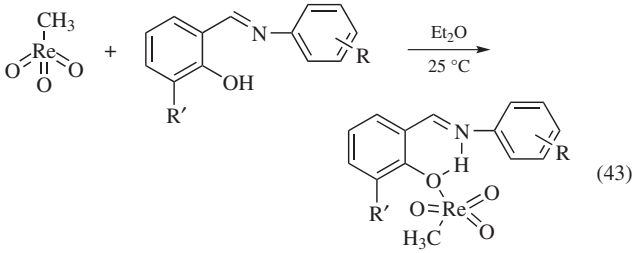
Epoxidation of Alkenes. The use of aromatic N-donor ligands in significant excess (ca. 10–12:1) together with MTO leads to higher activities and selectivities in epoxidation catalysis than with MTO alone.[12,15,16,18,56,57] This observation is made for both mono- and bidentate aromatic Lewis bases with N-donor ligands.[58–61] In the mean time, many N-ligand adducts of MTO have been isolated, characterized, and applied as catalysts for the epoxidation of olefins.[14,62–70] Other donor adducts of MTO, despite being mentioned sporadically in the literature, have never been examined to the same extent with respect to their applicability as epoxidation catalysts.[71] Rhenium complexes with Schiff base ligands derived from salicylaldehyde and mono- or diamines have received attention due to their applications in catalysis and nuclear medicine. Re oxo complexes bearing Schiff base ligands have been investigated extensively.[72–75] Kühn and coworkers described several Schiff base adducts of MTO (see eq 43) and applied them as epoxidation catalysts.[76,77] The yields of this system are between 20 % and 100 %.

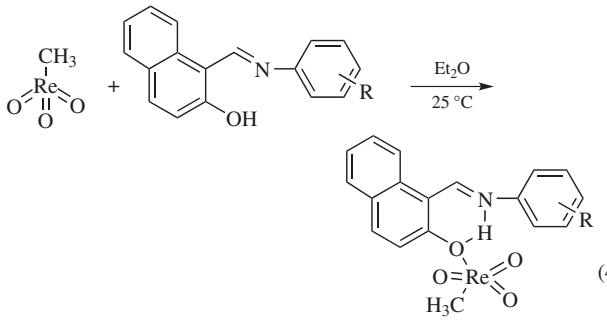


20–100% (43)

R′ = H, R = H; R′ = H, R = *p*-CH_3; R′ = H, R = *p*-Cl;
R′ = H, R = *p*-OCH_3; R′ = -OCH_3; R = *p*-CH_3

An -OCH_3 group on the Schiff base seems to destabilize the resulting complex under oxidative conditions, while other Schiff bases lead to active and highly selective epoxidation catalysts. An excess of ligand, however, always leads to rapid decomposition of the catalyst. The ready availability and stability of the Schiff base complexes, together with the good catalytic activity and high selectivity of some of them, make these materials good alternatives to less stable MTO/N-donor complexes as epoxidation catalysts. The Schiff base adducts of MTO can also be prepared and applied in situ. In contrast to N-donor adducts, no ligand excess is necessary to achieve high yields and selectivities in olefin epoxidation catalysis. In addition to the compounds shown in (eq 43),

other Schiff base complexes (eq 44) have been synthesized and investigated for catalytic activity.[78]

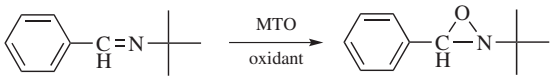


(44)

25–98%

R = H; R = *p*-CH_3; R = *o*-CH_3 + *m*-CH_3; R = *o*-Cl

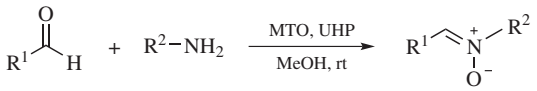
Oxidation of Aldimines. MTO was found to be an efficient catalyst for the oxygenation of various aldimines to the corresponding oxaziridines (eq 45) in excellent yields under mild conditions using solid peroxides such as urea hydrogen peroxide (UHP), sodium percarbonate (SPC), and sodium perborate (SPB) as oxidants. Among other rhenium-based catalysts studied, MTO was found to be the most efficient in this reaction. The use of solid peroxides offers nearly anhydrous medium, making the reactions more selective toward the oxaziridines by avoiding ring-opening products.[79]



(45)

98%

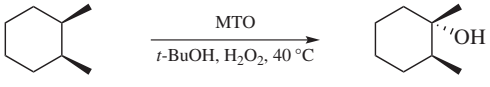
Oxidation of Primary Amines. Goti and coworkers described a one-pot condensation/oxidation of primary amines and aldehydes (eq 46) using UHP as stoichiometric oxidant in the presence of methyltrioxorhenium as catalyst. This reaction leads to nitrones in a simple and regioselective manner. From a sustainability point of view, this one-pot synthesis is simple to perform, takes place under mild conditions, and releases water as the only by-product.[80]



(46)

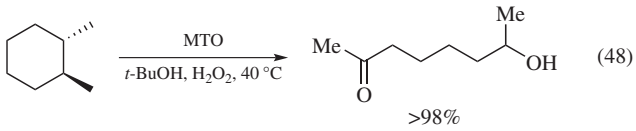
15–89%

Oxidation of Methyl-substituted Cyclohexane. Methyltrioxorhenium is a versatile catalyst for the oxidative functionalization of cyclohexane derivatives with H_2O_2 as oxygen donor. Interestingly, there is a difference between the two stereoisomeric *cis*- and *trans*-configurations (eqs 47 and 48).[81]



(47)

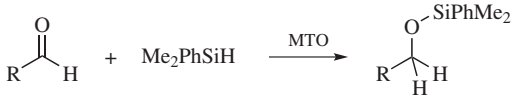
65%



(48)

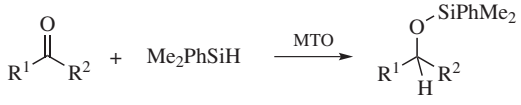
>98%

Hydrosilylation of Aliphatic and Aromatic Aldehydes. In 2003, Toste and coworkers reported the use of the dioxorhenium(V) derivative $[ReO_2I(PPh_3)_2]$ as catalyst for the hydrosilylation of aldehydes and ketones.[82] Based on these results, Romão and Royo investigated the activity of Re(VII) species in the same reaction. They pointed out that methyltrioxorhenium(VII) is the most active and versatile catalyst for the hydrosilylation of aliphatic and aromatic aldehydes (eq 49) and ketones (eq 50).[83]



(49)

41–96%

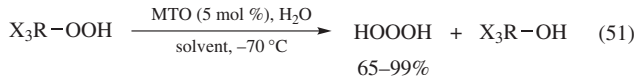


(50)

96–98%

Methyltrioxorhenium is an effective catalyst reaching a total conversion of the aldehyde to the silylated ether after 5 h at 80 °C.

Transformation of Hydrotrioxides. Hydrotrioxides (ROOOH) have already been found to be key intermediates in the reaction of ozone with alcohols, ethers, acetals, hydrocarbons, and organometallic hydrides.[84] Corey et al. have shown that triethylsilyl hydrotrioxide, $Et_3SiOOOH$, generated by the low-temperature ozonation of triethylsilane, is an excellent source of singlet oxygen ($O_2(^1\Delta_g)$).[85,86] Tuttle and coworkers found that under certain conditions, HOOOH is also formed in modest yields during the decomposition of some silyl hydrotrioxides in oxygenated organic solvents.[87] The same group reported that HOOOH is formed nearly quantitatively in the low-temperature MTO-catalyzed transformation of dimethylphenylsilyl hydrotrioxide in various solvents (acetone-d_6, methyl acetate, *tert*-butyl methyl ether) (eq 51). They found that other silyl hydrotrioxides, as well as some acetal hydrotrioxides investigated, react similarly.[88]



(51)

65–99%

R = Si, C; X = Me, Et, *n*-Bu, Ph

Second Update

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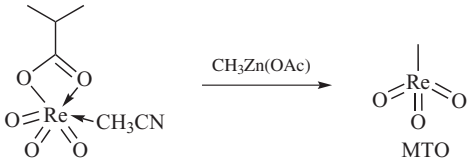
Max Planck Institute for Chemical Ecology, Jena, Germany

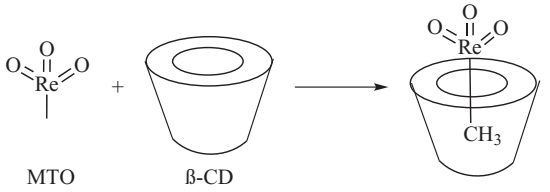
High Yield Nontoxic Synthesis. The reaction of methylzinc acetate with carboxyl perrhenates is an efficient and nontoxic way

of preparing MTO at an industrial scale.[89] High yields are obtained with acetyl- and isopropyl perrhenates (eq 52), yields 90% and 92%, respectively.



(52)

Epoxidation of Alkenes. Schiff and Lewis bases have been widely employed as ligands in the epoxidation of alkenes with MTO.[72–75,90–95] Qiu et al. evaluated several Schiff-base complexes of MTO for the epoxidation of cyclohexene. Strong Lewis basic ligands favored the metal–ligand interaction, while the catalytic activity and selectivity was only high with the urea hydrogen peroxide adduct (UHP) as an oxidant and poor with H_2O_2 (30%) due to its decomposition.[90] Halide substituted Schiff-bases have no important influence on the catalytic activity and selectivity.[93] Zhou et al. prepared epoxides from cyclooctene in high yield using hydrogen peroxide as an oxidant and bidentate Lewis base adducts of MTO as a catalyst. Selectivities for epoxide formation were higher than 99% and the stability of the bidentate complexes was superior compared to the monodentate ones.[96] Several attempts have been made to reduce decomposition of MTO in presence of H_2O_2. Al-Rawashdeh et al. covered the methyl group of MTO by the β-cyclodextrin cavity (see eq 53) minimizing catalyst decomposition and obtaining reasonable reactivity in basic media.[97] Polymer-supported/encapsulated MTO proved to be an efficient and selective system for the epoxidation of olefins.[98–100] Moreover, the more electron-rich double bond was selectively epoxidized as shown by Saladino et al. Catalysts were stable for at least four recycling experiments without loss of efficiency in terms of conversion and selectivity (TON = 37).[98]

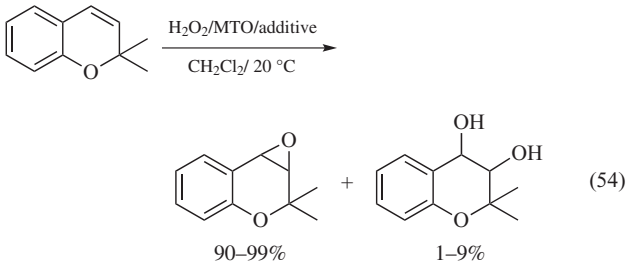


(53)

Chitin- and chitosan-anchored MTO were shown to be efficient catalysts for activation of UHP. Acid-sensitive double bonds were satisfactorily epoxidized with this system. *N*-functionalized chitosan was the most active system.[101]

New nitrogen-containing ligands and additives have been developed in recent years.[102–105] Michel et al.[103] employed *t*-butylpyridine for the selective epoxidation of (+)-limonene with H_2O_2 as an oxidant in a two-phase system. 1-Methylimidazole resulted in a suitable additive to avoid hydrolysis and rearrangement of acid-sensitive epoxides produced by MTO-catalyzed epoxidation (eq 54). Epoxides from styrenes, chromene, and bis(homoallylic) alcohols were obtained in good yields within reasonable reaction times.[104]

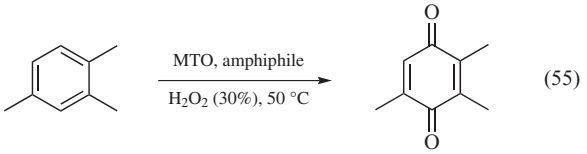
Ionic liquids proved to be excellent media for MTO-catalyzed epoxidation. A series of glycals were successfully epoxidized in the presence of 1-*n*-butyl-3-methylimidazolium hexafluorophosphate ([BMIM]PF_6) and 1-ethyl-3-methylimidazolium tetrafluoroborate with UHP as an oxidizing agent. Under these conditions, a higher stereoselectivity was observed than with H_2O_2 as the final oxidant.[106] The system MTO-Schiff-base/H_2O_2/[BMIM]PF_6 can be applied in alkene epoxidation under air.[107]



(54)

A CO_2-free ethylene oxide production was developed by Lee et al. using the H_2O_2/MTO system.[108] In this case, the selectivity was superior to the current industrial process. The absence of CO_2 makes this procedure eco-friendly. Another "green epoxidation" was carried out with H_2O_2 (35%)/MTO and 3-methylpyrazole as additive[109] under organic solvent-free conditions. This strategy avoids the use of the commonly employed dichloromethane and yields from 85% to 99% are obtained. Novel complexes of MTO with bis(fluorous-ponytailed)-2,2-bipyridines where tested to obtain high yields of epoxides, this study opened the path to explore fluorine chemistry in the MTO-based oxidation. The catalyst containing fluorine can be recovered by fluorous solid-phase extraction and reused in further runs.[110]

Aromatic Oxidation. Oxidation of pseudocumene with MTO-based catalysis has been reported.[111,112] The use of Lewis basic ligands has a beneficial effect on both, yield and selectivity, especially with (*N*-salicylidene)aniline-derived Schiff bases bearing electron-withdrawing substituents. Selectivity can be improved up to 80% when amphiphiles are used as additives (eq 55).[112]

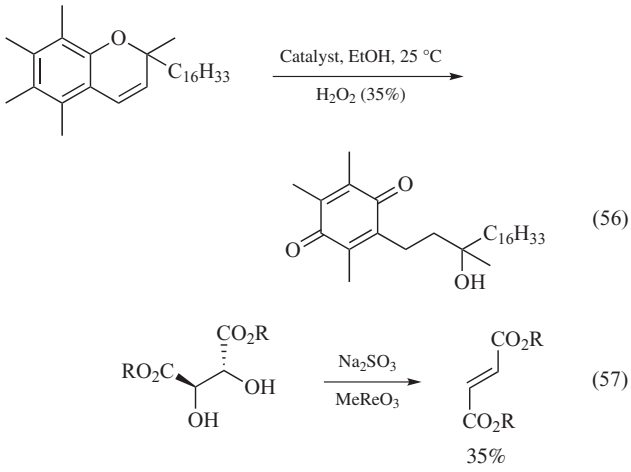


(55)

Tocopheryl quinones were efficiently prepared from the corresponding tocopherols by polymer-supported MTO/H_2O_2 catalytic system. Homogeneous MTO/H_2O_2 proved to be more reactive, but less selective than the heterogeneous catalyst, this may be explained by the presence of a kinetic barrier for the substrate to approach the supported rhenium complexes. Heterogeneous systems were stable to perform at least four recycling experiments with similar conversion and selectivity (eq 56).[113]

Deoxigenation of Diols and Epoxides. MTO was found to be a suitable catalyst for the reduction of vicinal diols and epoxides to the corresponding olefins employing H_2.[114] Triphenylphosphine[115] and sulfites have been also used as reducing agents to obtain moderate yields of olefins. MTO was more catalytically active, but less selective than ReO_4^- derivatives. The glycol configuration plays a key role in these reactions as *cis*- or *syn*-diols are

favored to undergo *syn*-elimination with retention of configuration of the double bond (eq 57).[116,117]

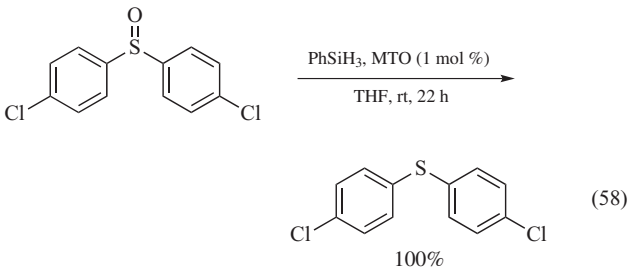


(56)

(57)

Hybrid Materials. Nanohybrid films of polyimide doped with Re^{7+} species had been prepared from MTO and polyamic acid via solution direct-dispersing method, with subsequent thermal imidization process.[118] MTO decomposes to ReO_2 during this treatment. The mechanical properties of the hybrid films can be improved by controlling the content of MTO.

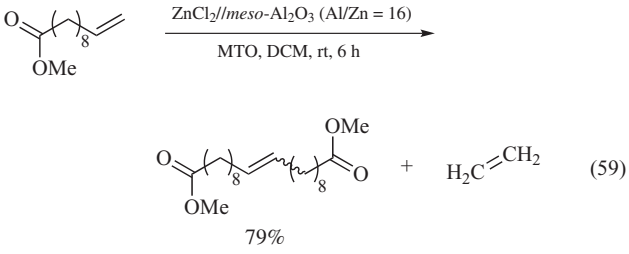
Reduction of Alkynes and Sulfoxides. The activation of hydrogen by Rhenium (V) and (VII) compounds had been reported by Reis et al. MTO selectively catalyzes hydrogenation of alkynes to alkenes at 80 °C and 40 atm with yields ranging from 47% to 100%.[119] Sousa and Fernandes reported the reduction of sulfoxides to sulfides catalyzed by MTO/silane complexes (eq 58), the reaction proceeded under mild conditions and with excellent yields for a variety of sulfoxides.[120]



(58)

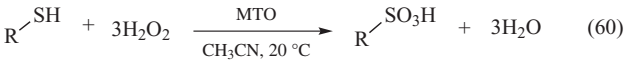
Similar conversions can be achieved with the system catecholborane/MTO.[121] The method allows an elegant and clean reduction of sulfoxides to sulfides, only a very low amount of catalyst (1 mol %) is needed and the reaction can be performed under air at room temperature.

Olefin Metathesis. The first efficient application of MTO as catalyst in olefins metathesis was described by Oikinawa et al. in 2007.[122] The authors modified mesoporous alumina with $ZnCl_2$ and employed it in the metathesis of olefins under mild conditions (eq 59). The effect of $ZnCl_2$ on the alumina surface was recently investigated by Tovar et al.; the authors showed that Zn is coordinated to the oxygen in the surface as well as to chloride. The overall effect of the Lewis acid resulted in an increase in the total number of active sites formed when MTO is deposited and an increase in the per-site activity due to interaction of MTO with Cl^- adsorbed on the surface.[123]



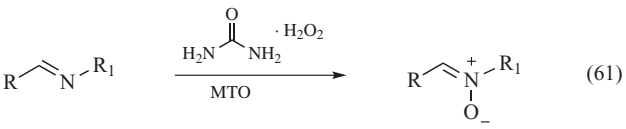
(59)

Oxidation of Thiols and Disulfides to Sulfonic Acids. Sulfonic acids have been prepared from the corresponding thiols with MTO/H_2O_2 as the oxidizing system. The protocol is simple, ecofriendly, and produces high yields (eq 60).[124] The same catalytic system was satisfactorily employed in the oxidation of diaryl- and dialkyl disulfides, in acetonitrile at 20 °C with high yields in short reaction times.[125]



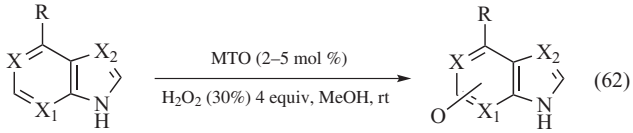
(60)

Oxidation of Nitrogen-containing Compounds. The MTO/UHP system has been used for *N*-oxidation of imines (eq 61).[126,127]



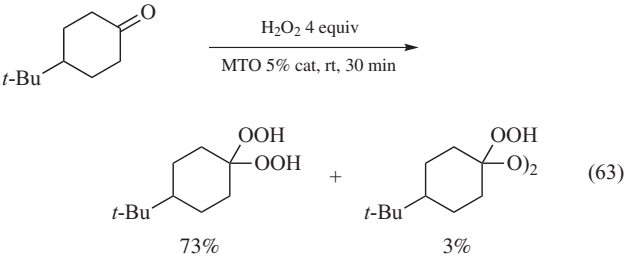
(61)

Methyltrioxorhenium also catalyzes the selective oxidation of purine and purine derivatives into their N-oxides (eq 62). Moderate to high yields can be obtained under mild conditions.[128,129]

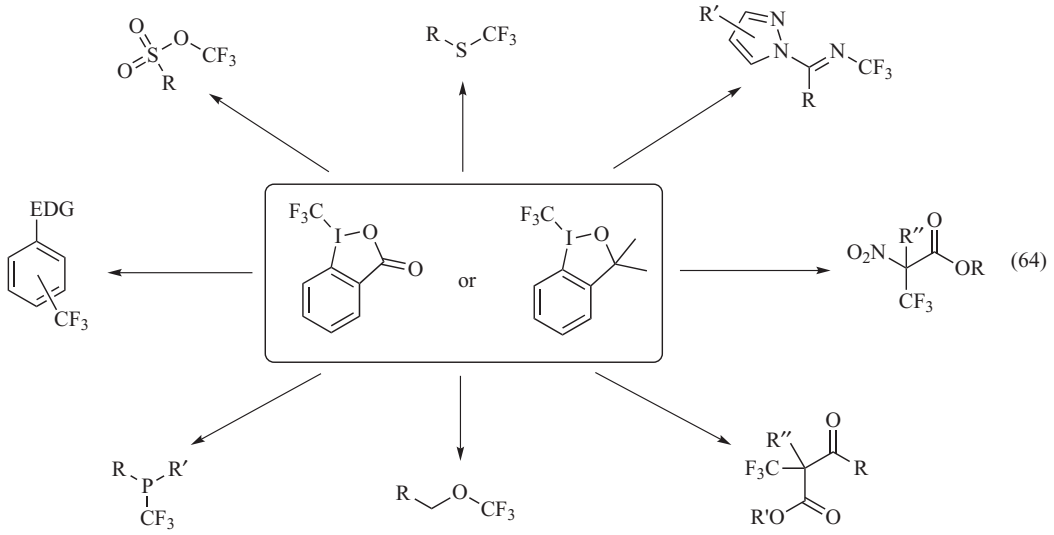


(62)

X, X_1, X_2 = N or CH;
R = H, Cl, Br, CH_3



(63)



Banerjee et al. performed *N*-oxidation of *meso*-tetraarylporphyrins and 2,3-dihydroporphyrins (chlorins) in good yields using the catalytic system MTO/H_2O_2/Pyrazole.[130] *Meso*-tetraphenylporphyrins were converted to the corresponding *N*-oxides in yields ranging from 50% to 80%, with ca. 20% recovery of the starting material. These results are similar to those obtained with peracids. Chlorin *N*-oxides are also produced in ~40% yield with ca. 50% recovery of the starting material, reactions should not be taken beyond 50% of conversion and stoichiometric quantities of MTO should be employed to avoid formation of side products. *Meso*-tetraaryldithiaporphyrins and chlorins can be *S*-oxidized with this method as well.

Miscellaneous Reactions. MTO finds an interesting application in the peroxyacetalization of ketones, aldehydes, and acetals with H_2O_2 as the oxidant, 1,1-dihydroperoxides are obtained (eq 63).[131]

Mejía et al.[132] achieved trifluoromethylation of arenes and heteroarenes by hypervalent iodine reagents with MTO as a catalyst (5–10 mol %) and yields up to 77% (eq 64).

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9. General discussion

My research provides new insights into the JA-Ile signaling pathway in both early events triggering JA biosynthesis and late events like the accumulation of defense metabolites. Besides generating new knowledge in the JA perception and shutdown mechanisms, I have explored the fluorine chemistry as a new potential tool to study the role of JAs in plants.

Each relevant outcome of my work has been discussed in detail in the corresponding section of this thesis. Therefore, the aim of this chapter is to place the results previously presented in a broader and more general context. Furthermore, I mention some aspects which are suggested by my research and should be considered for future investigations in this field.

9.1. Calcium-dependent jasmonate signaling

In plant-herbivore interactions the very early events of recognition of elicitors and signal transduction to downstream induced defenses (mainly controlled by phytohormones such as JAs) are poorly understood. Ion fluxes at the plasma membrane are deeply involved in the activation of defense-related genes, ROS burst and protein kinase activation downstream to the elicitor recognition [106,107]

The Ca^{2+} cation is a universal second messenger which plays an important role as a mediator for responses against several biotic and abiotic stimuli [7,9]. Changes in $[Ca^{2+}]_{cyt}$ are one of the earliest signaling events observed in plant-herbivore interactions after MAMP/PAMP perception [7]. On the one hand, the cascade of reactions following increases in $[Ca^{2+}]_{cyt}$ concentration were reported to directly impact TFs involved in defense responses independent of phytohormone-related signaling pathways [108]. On the other hand, Ca^{2+} can also affect downstream herbivore-triggered signaling cascades related to hormones like JAs, as Ca^{2+} elevations are induced by these compounds and some derivatives in *N. benthamiana* BY-2 cell culture [109].

As stated in the introductory part of this dissertation, evidence suggests that Ca^{2+} and JA signaling pathways are closely related. Further evidence of the close relationship between Ca^{2+} and JAs was provided in the manuscript “Neomycin

inhibition of (+)-7-*iso*-jasmonoyl-L-isoleucine accumulation and signaling" discussed in section 4. It was demonstrated that neomycin, a Ca^{2+} inhibitor, blocks OS-induced $[Ca^{2+}]_{cyt}$ elevation and interferes with JA-Ile accumulation and signaling in *A. thaliana*. Interestingly, JA accumulation was not affected by neomycin pre-treatment and the expected JA burst was observed. The expression of the marker JRGs, *VSP2* and *LOX2* was down regulated in neomycin treated plants in correspondence with the inhibition of JA-Ile accumulation. A higher expression of the P450 enzyme CYP94B3 gives a plausible explanation for the lower contents of JA-Ile found in neomycin treated plants. A higher level of this enzyme seems to be responsible for a faster JA-Ile turnover. In this work, neomycin is introduced as a valuable tool to study Ca^{2+}-signaling in plants, especially in those species where the absence of mutants can be an important drawback to deal with.

Inositol polyphosphates (InsP) may be the missing link between Ca^{2+} and JA-Ile signaling. Neomycin inhibits the production of inositol-1,4,5-trisphosphate by acting on PLC (phospholipase C) in *A. thaliana*, which is thought to affect the internal Ca^{2+} store levels [110]. It has been demonstrated that InsP are deeply involved in JA-Ile perception [67,68]. Therefore, the relationship Ca^{2+}/InsP and its influence in JA-Ile perception and signaling should be an important topic to investigate in the future. Currently, I explore the role of inositol pentakisphosphate in JA-Ile signaling with the help of molecular modeling (see chapter 7).

9.2. Fluorine chemistry as a potential tool to study jasmonates metabolism

The mobile signal(s) which activates plant defenses in parts distant from the damaged areas (systemic response) has remained elusive. The activation of systemic defenses against diverse stimuli is crucial to the plant development. Molecules like the 18-amino acid polypeptide systemin [111] and phytohormones (e.g. JA and SA), together with the most recently developed squeeze-cell hypothesis [112], have being associated with the induction of systemic defense responses in different plant species.

Currently, it is widely accepted that JAs are deeply involved in systemic plant responses [30]. It has been demonstrated that the close relationship between Ca^{2+} and JA signaling occurs not only in local leaves but also in systemic ones. Wounding and herbivory trigger fast $[Ca^{2+}]_{cyt}$ spikes in systemic leaves with direct vascular connections to the local damaged leaf [113], while salt stress-induced Ca^{2+} waves rapidly propagate from root-to-shoot in plants [114]. Such events are accompanied by an increase in the internal levels of jasmonates. Furthermore, it has been shown that mechanical wounding activates *de novo* biosynthesis of JAs in leaves distal to wounds, and that JAs can be systemically translocated [30,31,115,116].

During my research I synthesized a fluorinated analogue of the JA precursor OPC-8:0 (7F-OPC-8:0). I successfully employed 7F-OPC-8:0 as a probe to study its transport in *A. thaliana* plants. I showed that this compound is translocated from damaged leaves to undamaged systemic leaves, suggesting that the JA precursor OPC-8:O – and not only JA and JA-Ile – can also contribute to the activation of systemic plant defenses. 7F-OPC-8:0 induces the expression of marker JRG and the accumulation of endogenous jasmonates in *A. thaliana* leaves. Furthermore, it is possible to detect 7F-OPC-8:0 derived metabolites (produced after its β-oxidations) in leaves extracts. This suggests that 7F-OPC-8:0 is a true mimic of the endogenous compound and can be metabolized to JAs downstream in the JA-pathway and consequently activate gene expression.

To my knowledge, it is the first time that systemic translocation of a mimic of OPC-8:0 has been demonstrated. This finding also implies that translocated JA-precursors may contribute to the total JAs accumulation observed in systemic leaves of different plant species. Moreover, my results exalt the potential of the fluorine-based analytical chemistry to study jasmonates – and likely other plant metabolites – metabolism and signaling. Additionally, plants are the energy source of many herbivorous organisms, therefore fluorinated JAs may be employed to study the metabolic fate of these molecules in feeding organisms or even in tri-trophic interactions. Fluorine is a monoisotopic element with a long lived radioactive isotope (^{18}F) and high gyromagnetic ratio (γ = 40.05 MHz/T). These properties make fluorinated JAs interesting compounds to be employed in techniques like HRMS, PET and ^{19}F-NMR. For instance, the replacement of the

^{19}F atom by its radioactive isotope ^{18}F in 7F-OPC-8:0 will allow investigating the transport of this molecule in real time employing PET.

9.3. Structure activity relationship of jasmonates. Manipulation of the JA-signaling pathway

Since the discovery and crystal structure elucidation of the jasmonate receptor (SCF^{COI1}) only a few studies dealt with SAR of JAs. In the 1980s-90s, however, several studies on SAR of JAs where carried out from a chemical perspective. First studies conducted to understand the mode of action and inactivation of JAs showed divergences in the structural requirements for the effects of these molecules on plant growth, tuber-inducing capability, leave senescence, seed germination and alkaloids production (see section 1.1.2.5). In recent years, important progress has been made understanding the biological processes involved in the JA-Ile signaling pathway. Several enzymes involved in the JA-Ile biosynthesis, perception and catabolic mechanisms have been identified and well characterized [27]. Nevertheless, the JA-Ile signaling pathway is yet an exciting field of research.

The idea of tailoring JAs for eliciting particular responses in the plant (i.e. manipulate the JA-signaling pathway) has been around for several years. Based on the vast information available about the JA-Ile receptor, including its x-ray structure, I carried out several studies on SAR of the biologically active jasmonate JA-Ile. I hypothesized that a lactone such as the JA-Ile-lactone may exist in nature in analogy to the Jasmine ketolactone (see figure 1, article III). Such a lactone contains the important moieties required for jasmonate perception and therefore may be biologically active. Two diastereomeric JA-Ile-lactones showed strong biological activity regarding the induction of nicotine accumulation in the leaves of *N. attenuata* plants (chapter 6, Article III). These exiting results prompted me to move from *N. attenuata* to the robust model system *A. thaliana*. The existence of *A. thaliana* knockout mutants and the known crystal structure of the JA-Ile receptor in this species would facilitate the design of experiments and the data interpretation.

I designed and synthesized 12-modified JA-Ile derivatives to explore the inactivation mechanism of JA-Ile via the ω-hydroxylation pathway. I employed gene expression assays of marker JRGs as positive control for the activity of the synthetic compounds. Furthermore, modeling studies (docking) of the rationally-designed derivatives with the receptor complex (SCF^{COI1}) are being studied currently.

In this thesis I proposed that the inactivation of the bioactive jasmonate JA-Ile upon ω-hydroxylation is likely to be caused by the steric hindrance of the ─OH group (chapter 7). The enlargement of the pentenyl side chain of JA-Ile (e.g. by introduction of a hydroxyl group at C12) negatively affects the recognition of the molecule by the SCF^{COI1} receptor complex. An interesting outcome of my study was that a free carboxyl group seems to be not essential for the interaction of JA-Ile analogues (e.g. coronalon) with the SCF^{COI1} receptor complex, whereas an ester bond seems to be sufficient.

9.4. Concluding remarks and outlook

Several discoveries have shed light on our understanding of JA-signaling in the last few years. JA-Ile was identified as the bioactive endogenous jasmonate [57,84], and its receptor complex was revealed [66,88]. JAZ repressors were stablished as substrates of the SCF^{COI1} receptor complex [61,62,117], which crystal structure was recently determined [67]. NINJA and TOPLESS were identified as important interactors of JAZs [69]. Additionally, many TFs activated in a JA-Ile-dependent manner have been described [118-120]. However, despite recent advances in this field, several aspects of JA-signaling have remained unclear.

In my work data supporting the close relationship between Ca^{2+} and the JA-Ile signaling pathways was generated. Further studies in this area should be addressed to unravel key players involved in the maintenance of Ca^{2+} homeostasis in the cell after herbivory attack. Within this context the relationship among Ca^{2+} sensing proteins such as CMLs and JA-Ile signaling should be studied.

In this dissertation I carried out pioneer studies on the fluorine chemistry of JA derivatives. The unique properties of fluorine can now be employed to further explore JA-signaling. For instance, fluorinated JAs should be prepared to explore their biological activity by fluorine based techniques like ^{19}F-NMR and PET in real time.

JA-Ile is accepted as the bioactive ligand that promotes COI1-JAZ interactions. Nevertheless, other JA-amino acid conjugates also promote COI1-JAZ interactions *in vitro* pull down experiments [66]. I have proved that JA-Ile-lactones induce typical jasmonate-related responses in different plant species. Therefore, it would be interesting to investigate how these molecules promote COI1-JAZ interactions and whether similar lactones from other JA-amino acid conjugates exhibit any other biological properties as well. Equally interesting would be to compare the gene responses (genome wide) induced by the JA-Ile-lactones with responses elicited by the endogenous JA-Ile. This approach may reveal transcriptional differences generated by the architecture of these molecules. Finally, I want to mention that the optimization or development of new inexpensive synthetic routes to rationally designed JAs may facilitate the use of these compounds in agriculture to enhance crop protection.

With my dissertation I contributed to the understanding of the mechanism of action of jasmonates. The results presented here shed light on the JA-Ile signaling pathway and therefore on plant-insect herbivore interactions. Future studies in this field will help to elucidate how plants, upon JAs, balance growth and defenses in response to a manifold of environmental conditions.

10. Bibliography

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11. Appendix I: Publications within the PhD period, but not related to the main topic of the dissertation

11.1. Article V: Improved synthesis of the chrysomelid pheromone (6R,7S)-(+)-himachala-9,11-diene via spontaneous bromination and didehydro-bromination of 2,6,6,9-tetramethyl-bicyclo[5.4.0]undec-8-ene

A convenient synthesis of (6*R*,7*S*)-(+)-himachala-9,11-diene, the pheromone of the chrysomelid beetle *Phyllotreta striolata* is described. The diene is obtained in a single operation by a spontaneous "bromination/dehydrobromination" of 2,6,6,9-tetramethylbicyclo[5.4.0]undec-8-ene. The halogenation/dehalogenation sequence proceeds spontaneously in CCl_4, and is less uniform in CH_2Cl_2 and $CHCl_3$. ^1H NMR experiments carried out in presence of the radical scavenger ditert-butyl-4-methylphenol suggest an ionic mechanism for this reaction. Theoretical calculations demonstrate that the spontaneous reaction profits from the strongly exergonic addition of Br_2 to the double bond and an almost neutral energy difference between the starting olefin and the diene pheromone.

Publikation	Improved synthesis of the chrysomelid pheromone (6*R*,7*S*)-(+)-himachala-9,11-diene via spontaneous bromination and didehydro-bromination of 2,6,6,9-tetramethyl-bicyclo[5.4.0]undec-8-ene. Guillermo H. Jimenez-Aleman, Tim Schöner, Ana L. Montero-Alejo, Wolfgang Brandt, Wilhelm Boland. *Arkivoc*. **2012**, (iii) 371-378.				
Beteiligt an	G.H. Jimenez-Aleman	T. Schöner	A.L. Montero-Alejo	W. Brandt	W. Boland
Konzeption des Forschungsansatzes	X				X
Planung der Untersuchungen	X				X
Datenerhebung	X	X	X	X	
Datenanalyse und -interpretation	X		X	X	X
Schreiben des Manuskripts	X				
Vorschlag Anrechnung Publikationsäquivalente	— Do not apply. (contributed ~ 80%)				

11.2. Article VI: Behavioral responses of *Phyllotreta striolata* to sesquiterpenoid pheromone components and allyl isothiocyanate

Aggregations of the flea beetle *Phyllotreta striolata* on their crucifer host plants are mediated by a pheromone which is emitted by males. The male-specific sesquiterpene (+)-(6*R*,7*S*)-himachala-9,11-diene (**1**) was shown to be physiologically and behaviorally active, but to be attractive under field conditions, the concomitant presence of the host plant volatile allyl isothiocyanate (AITC) is required. Here we analyzed the behavioral response of *P. striolata* adults to the male-specific compound (3*S*,9*R*,9a*S*)-3-hydroxy-3,5,5,9-tetramethyl-5,6,7,8,9,9a-hexahydro-1H-benzo[7]annulen-2(3H)-one (**2**) and further investigated the interaction of the aggregation pheromone **1** with AITC. Compound **2** alone was attractive to *P. striolata* in laboratory assays; however, significantly more beetles were attracted if this compound was combined with **1**. Furthermore, traps baited with the combination lure also attracted significantly more *P. striolata* adults than control traps in field experiments. AITC was previously shown to be attractive to *P. striolata* adults in high doses exceeding the natural emission rate, and **1** further enhanced its attractiveness. In field trapping experiments we found no attraction to natural concentrations of AITC, and it did not enhance the behavioral response to the male-specific sesquiterpenoids. *P. striolata* adults even preferred the sesquiterpenoid lure alone over the blend containing the highest dose of AITC (1 mg per trap) tested in laboratory assays. In conclusion, we show that the male-specific compound **2** is part of the male-produced aggregation pheromone. Although AITC is an attractant at high doses, our data indicate that AITC does not play a crucial role in mediating aggregation behavior of *P. striolata* adults on host plants.

Publikation	Behavioral responses of *Phyllotreta striolata* to sesquiterpenoid pheromone components and allyl isothiocyanate. Franziska Beran, Guillermo H. Jimenez-Aleman, Ramasamy Srinivasan, Inga Mewis, Christian Ulrichs, Wilhelm Boland, Bill s. Hansson, Andreas Reinecke. (in preparation)							
Beteiligt an	F. B	G.H. J-A	R. S	I. M	C. U	W. B	B. H	A. R
Konzeption des Forschungsansatzes	X		X	X	X		X	X
Planung der Untersuchungen	X	X				X		
Datenerhebung	X	X						
Datenanalyse und -interpretation	X							X
Schreiben des Manuskripts	X							X
Vorschlag Anrechnung Publikationsäquivalente	— Do not apply. (contributed ~ 15%)							

11.3. Article VII: Foliar endophytic fungi as potential protectors from pathogens in myrmecophytic Acacia plants.

In defensive ant-plant interactions myrmecophytic plants express reduced chemical defense in their leaves to protect themselves from pathogens, and it seems that mutualistic partners are required to make up for this lack of defensive function. Previously, we reported that mutualistic ants confer plants of *Acacia hindsii* protection from pathogens, and that the protection is given by the ant-associated bacteria. Here, we examined whether foliar endophytic fungi may potentially act as a new partner, in addition to mutualistic ants and their bacteria inhabitants, involved in the protection from pathogens in myrmecophytic Acacia plants. Fungal endophytes were isolated from the asymptomatic leaves of *A. hindsii* plants for further molecular identification of 18S rRNA gene. Inhibitory effects of fungal endophytes were tested against Pseudomonas plant pathogens. Our findings support a potential role of fungal endophytes in the protection mechanisms against pathogens in myrmecophytic plants and provide the evidence of novel fungal endophytes capable of biosynthesizing bioactive metabolites.

Publikation	Foliar endophytic fungi as potential protectors from pathogens in myrmecophytic Acacia plants. Marcia González-Teuber, Guillermo H. Jimenez-Aleman, Wilhelm Boland. *Communicative & Integrative Biology*, 2014,7:5, 1-4. DOI:10.4161/19420889.2014.970500		
Beteiligt an	M. González-Teuber	G.H. Jimenez-Aleman	W. Boland
Konzeption des Forschungsansatzes	X		
Planung der Untersuchungen	X	X	
Datenerhebung	X	X	
Datenanalyse und -interpretation	X	X	X
Schreiben des Manuskripts	X		
Vorschlag Anrechnung Publikationsäquivalente	─ Do not apply. (contributed ~ 40%)		

11.4. Article VIII: Investigation of gossypols toxicity to Heliothine larvae (*Helicoverpa armigera*). A Schiff base Gossypol and the candidate enzyme CYP6AE14

Gossypol is a polyphenolic secondary metabolite produced by cotton plants. Due to its chemical structure this compound results toxic to many organisms. The aldehyde groups of gossypol are supposed to especially contribute to its toxicity. Although gossypol has been extensively studied in mammals for its antifertility, antitumor, antimalarial, antiamoebic, antiparasitic, and antiviral activities, very little is known about its mode of action/toxicity to cotton-feeding insects. Here we test the toxicity of a gossypol derivative (where the aldehyde groups have been replaced by an imine) towards the generalist herbivore *Helicoverpa armigera*, which is an important pest on cotton plants. Additionally, we investigated the capability of the gene/enzyme CYP6AE14 ─ which is claimed to be involved in gossypol detoxification in *H. armigera* larvae ─ to metabolize gossypol. Larval feeding studies with *H. armigera* on artificial diet supplemented with the modified gossypol, gossypol and no gossypol as a control diet revealed no significant difference in larval net weight gain when larvae fed on modified gossypol and control diet. Only larvae fed on gossypol supplemented diet showed a reduced larval net weight gain. Further we successfully heterologously expressed CYP6AE14 in insect cell lines. In incubation assays with microsomal CYP6AE14 and gossypol, no gossypol derived metabolites were detected. qPCR data of

larvae feeing on several other plant toxins showed a CYP6AE14 up regulation on almost all experiments.

Publikation	Investigation of gossypols toxicity to Heliothine larvae (*Helicoverpa armigera*): A Schiff base Gossypol and the candidate enzyme CYP6AE14. Corinna Krempl, Nicole Joussen, Guillermo H. Jimenez-Aleman, Michael Reichelt, Hanna Heidel-Fischer, Heiko Vogel and David G. Heckel. (in prepapration)						
Beteiligt an	C. Krempl	N. Joussen	G.H. J-A	M. Reichelt	H.H. Fischer	H. Vogel	D.G. Heckel
Konzeption des Forschungsansatzes	X	X			X	X	X
Planung der Untersuchungen	X		X				
Datenerhebung	X		X	X			
Datenanalyse und -interpretation	X	X		X		X	
Schreiben des Manuskripts	X						
Vorschlag Anrechnung Publikationsäquivalente	— Do not apply. ~ 10%						

12. Selbstständigkeitserklärung

Erklärung:

Ich erkläre,

dass ich die vorliegende Arbeit selbständig und unter Verwendung der angegebenen Hilfsmittel, persönlichen Mitteilungen und Quellen angefertigt habe,

dass mir die geltende Promotionsordnung der Chemisch-Geowissenschaftlichen Fakultät bekannt ist;

dass ich die Dissertation selbst angefertigt und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben habe;

dass mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: siehe Danksagung;

dass die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen;

dass ich die Dissertation noch nicht als Prüfungsarbeit für staatliche oder andere wissenschaftliche Prüfungen eingereicht habe;

dass ich nicht die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena,

Guillermo Hugo Jimenez Aleman

13. Acknowledgements

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14. Curriculum Vitae

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Place of birth: Morón, Ciego de Ávila, Cuba.
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Marital status: Married (2 children)

EDUCATION

1993-1996: ***High School of Exact Sciences*** *"Ignacio Agramonte"*, Ciego de Ávila, Cuba.

1997-2002: Degree in Chemistry (10 semesters) at the University of Havana (UH), Cuba. (GPA: 4,92 / 5) Degree with honors.

Diploma thesis: "Introduction of an immunochemical label in nucleosides analogues for non-radioactive detection of DNA sequences" (Mention: Highest Honor). Department of Molecular Biology, National Center of Neuroscience, Havana. Cuba.

Supervisors: Dr. Chryslaine Rodríguez-Tanty, National Center of Neuroscience. Department of Molecular Biology. Havana, Cuba.

Prof. Dr. Ricardo Martínez-Sánchez, Institute of Science and Technology of Materials (IMRE), UH, Cuba.

SCIENTIFIC TRAINING

12/2001-06/2002: Synthetic chemistry: Diploma thesis. Department of Molecular Biology. National Center of Neuroscience. Havana, Cuba.

10/2002-10/2009: Polymer chemistry/Synthetic chemistry: Staff Researcher at IMRE, UH, Cuba.
Supervisor: Prof. Dr. Ricardo Martínez-Sánchez, Director of the Department of Polymer Chemistry, IMRE, UH, Cuba.

09/2006-10/2009: PhD student at the Department of Polymer Chemistry, IMRE, UH, Cuba. Study of the mechanism of polymerization of furfuryl alcohol: Hybrid materials of silica gel and furanic monomers. (Interrupted due to personal reasons)

11/2009-11/2010: Paternity leave (twins). I moved from Cuba to Germany permanently.

12/2010- to present: PhD student at the Department of Bioorganic Chemistry, Max Planck Institute for Chemical Ecology (MPI-CE) and Friedrich-Schiller-University of Jena, Germany.
PhD thesis: Synthesis of jasmonates and derivatives to study plant signaling: Activation, translocation and shutdown mechanisms.

LAB/ TECHNICAL SKILLS

- Organic synthesis
- Analytical chemistry. UV/Vis, FTIR, NMR (Conventional 1D and 2D, and solid state), chromatography (TLC, preparative, GC-MS, LC-MS/MS), x-ray
- Chemical ecology (plant signaling, phytohormones crosstalk, plant-insect interactions)
- Metabolomics
- RT-PCR
- Polymer chemistry
- Material science
- SEM, TEM, AFM, STM Microscopy

LANGUAGES

Spanish	Native language
English	Fluent
German	Fluent

RELEVANT ATTENDED COURSES

- Introduction to real-time PCR. Institute of Pharmacy, FSU Jena. Germany
- Plant secondary metabolites. Biochemistry Department. MPI-CE. Jena. Germany
- Introduction to microscopy. Department of Evolutionary Neuroethology. MPI-CE. Jena. Germany
- Analysis of small molecules by LC-MS. ITB Department. MPI-CE. Jena. Germany
- Advanced mass spectrometry. MPI-CE. Jena. Germany
- Statistics. MPI-CE. Jena. Germany.
- Synthesis and Mechanism of Polymerization. Faculty of Chemistry. University of Havana.
- 2D NMR Spectroscopy. Faculty of Chemistry. University of Havana.
- Optical and Electron Microscopy. IMRE. University of Havana.
- Electron microscopy and microanalysis. Institute of Geosciences, FSU Jena. Germany

AWARDS

- Gesellschaft Deutscher Chemiker e.V.: Travel Award
- International Society of Chemical Ecology: Travel Award

RELEVANT PRESENTATIONS

1. **Jiménez-Alemán G. H.:** JA-Ile-Macrolactones induce nicotine accumulation in *Nicotiana attenuata* leaves and reduce *Manduca sexta*

mass gain and survivorship. *31st International Society of Chemical Ecology meeting Stockholm, June 30th,* ***2015****.* Student travel award winner. *(oral)*

2. **Jiménez-Alemán G. H.:** "JAZ"Z played by Jasmonates: Is one instrument enough to form the Orchestra? *Institute Symposium, September* ***2014****. Max Planck Institute for Chemical Ecology, Jena, Germany. (oral)*

3. **Jiménez-Alemán G. H.:** New Insights into Structure-Activity Relationships and metabolism of Jasmonates**.** *30th annual International Society of Chemical Ecology meeting, July* ***2014****. University of Illinois, Urbana-Champaign, Illinois, USA. (oral)*

4. **Jiménez-Alemán G. H.:** Jasmonates: New Insights into Structure-Activity Relationships and Metabolism. *The Chemistry and Biology of Natural Products Symposium VIII, May* ***2014****. University of Warwick, UK. (oral)*

5. **Jiménez-Alemán G.H.:** How do plants shut down jasmonate signalling? *12th IMPRS Symposium, Apr* ***2012****. Max Planck Institute for Chemical Ecology, Jena, Germany. (poster)*

6. **Jiménez-Alemán G.H.:** Synthesis of the chrysomelid pheromone (6R,7S)-(+)-himachala-9,11-diene via spontaneous bromination and didehydrobromination of 2,6,6,9-tetramethylbicyclo-[5.4.0]undec-8-ene. *European-Winter School on Physical Organic Chemistry (E-WISPOC 2012), February* ***2012****. Bressanone, Italy. (poster)*

7. Rodríguez-Tanty C., Pérez D., Higginson D., Sablón M., Riveron A. M., **Jiménez-Alemán G.H.**, Velez H., Fernández A., Zayas M., Diaz N., Perez R.: Non–Radioactive Labeling of DNA for the Disease Diagnostic. *1st Latinoamerican Meeting of Medicinal Chemistry, April* ***2007****. Montevideo. Uruguay. (poster)*

PUBLICATIONS

1. **Guillermo H. Jimenez-Aleman**, J. Vadassery, A. Mithöfer, W. Brandt and W. Boland. Steric hindrance as key factor for inactivation of JA-Ile after ω-hydroxylation. *(in preparation)*

2. Franziska Beran, **Guillermo H. Jimenez-Aleman**, Ramasamy Srinivasan, Inga Mewis, Christian Ulrichs, Wilhelm Boland, Bill s. Hansson, Andreas Reinecke. Behavioral responses of *Phyllotreta striolata* to sesquiterpenoid pheromone components and allyl isothiocyanate. *(in preparation)*

3. Corinna Krempl, Nicole Joussen, **Guillermo H. Jimenez-Aleman**, Michael Reichelt, Hanna Heidel-Fischer, Heiko Vogel and David G. Heckel. Investigation of gossypols toxicity to Heliothine larvae (*Helicoverpa armigera*). A Schiff base Gossypol derivative and the candidate enzyme CYP6AE14. *(in preparation)*

4. **Guillermo H. Jimenez-Aleman**, Sandra S. Scholz, Monika Heyer, Michael Reichelt, Axel Mithöfer, and Wilhelm Boland. Synthesis, metabolism and systemic transport of a fluorinated mimic of the endogenous jasmonate precursor OPC-8:0. *Biochimica Et BBA-Mol. Cell Biol. L.*, **2015**, *1851*, 1545-1553.

5. **Guillermo H. Jimenez-Aleman**, Ricardo A. R. Machado, Helmar Görls, Ian T. Baldwin and Wilhelm Bolan. Synthesis, structural characterization and biological activity of two diastereomeric JA-Ile macrolactones. *Org. Biomol. Chem.*, **2015**, *13*, 5885. (front cover)

6. Marcia González-Teuber, **Guillermo H. Jiménez-Alemán** and Wilhelm Boland. Foliar endophytic fungi as potential protectors from pathogens in myrmecophytic Acacia plants. *Commun. Integr. Biol.*, **2014**, *7*, 1-4.

7. Jyothilakshmi Vadassery, Michael Reichelt, **Guillermo H. Jiménez-Alemán**, Wilhelm Boland and Axel Mithöfer. Neomycin Inhibition of (+)-7-iso-Jasmonoyl-L-Isoleucine Accumulation and Signaling. *J. Chem. Ecol.*, **2014**, *40*, 676–686.

8. **Guillermo H. Jimenez-Aleman** and Wilhelm Boland. Methyltrioxorhenium (VII). Second Update. *Encyclopedia of Reagents for Organic Synthesis*. *e-EROS.*, **2013**, 12-17. Chichester: Wiley. DOI: 10.1002/047084289X.rn00017.pub3

9. **Guillermo H. Jimenez-Aleman,** Tim Schöner, Ana L. Montero-Alejo, Wolfgang Brandt and Wilhelm Boland. Improved synthesis of the chrysomelid pheromone (6*R*,7*S*)-(+)-himachala-9,11-diene via spontaneous bromination and didehydrobromination of 2,6,6,9-tetramethyl-bicyclo[5.4.0]undec-8-ene. *Arkivoc*, **2012**, *3*, 371-378.

10. Martha Príncipe, Heidi Suárez, **Guillermo H. Jimenez-Aleman,** Ricardo Martínez and Stefan Spange. Composites prepared from silica gel and furfurylalcohol with *p*-toluenesulfphonic acid as the catalyst. *Polym. Bull.*, **2007**, *58*, 619.

11. David Higgison, Dannelys Pérez-Bello, Ana María Riverón-Rojas, Esteban Gutiérrez, **Guillermo H. Jimenez-Aleman,** Mirta Zayas, Rafaela Pérez Marquiza Sablón, Nelson Díaz, Chryslaine Rodríguez-Tanty. Non-Enzymatic in vitro DNA labelling and label inmunoquantification. *Prep. Biochem. Biotech.*, **2005**, *35*, 1-15.

12. Marquiza Sablón, Chryslaine Rodríguez-Tanty, Rafaela Pérez, **Guillermo H. Jimenez-Aleman,** Danay Dupeyron. Synthesis and characterization of non-radioactive labels derivatives from naphthalene. *Revista Cubana de Química*, **2004**, *16*, 864.

13. **Guillermo H. Jimenez-Aleman,** Hermán Vélez, Rafaela Pérez, Marquiza Sablón, Nelson Díaz, Chryslaine Rodríguez-Tanty. Synthesis of new analogues of thymidine for non-radioactive detection of DNA sequences. *Revista Cubana de Química*, **2004**, *16*, 1201.